VARIATION IN VACCINIA VIRUS.

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A thesis submitted for the degree of M.Sc. in the Australian National University, Canberra.

CONTENTS.

STATEMENT.

The work reported in this thesis was carried out while the candidate was a Departmental Assistant in the Department of Microbiology.

The experiments described were performed by the candidate, unless otherwise indicated in the text, under the supervision of Professor F. Fenner. The work in Part II was carried out under direct supervision, while that in Part III was done along general lines suggested by Professor Fenner.

A. Gemmell.

PREFACE.

The scope implied by the title of this thesis is immense, as it covers almost all examples of alteration in the biological behaviour of vaccinia virus. No attempt will be made to cover this field completely. After a brief description of known mechanisms of virus variation (Part I), attention will be concentrated on two aspects of the problem; first the adaptation of vaccinia virus to the mouse brain and second the behaviour and properties of the white variants of vaccinia virus.

Vaccinia virus has been adapted to the rabbit and mouse brain by many workers in the past, but there has been no profitable study of the mechanisms of this process of adaptation. The technique of titrating vaccinia virus on the chorioallantoic membrane provides a potential method for studying the changes in the structure of a virus population as it undergoes adaptation. It was hoped that its use might show the operation of mutation and selection during the process of adaptation. In the event it did not prove possible to carry this investigation to completion with the two strains used, for the virulent and a-virulent components of the virus populations in these cases showed no definite differences in pock morphology.

The second stage of this work was concerned with the white variants of vaccinia virus. These variants are of considerable

interest since they occur with very high frequency, suggesting that a white pock may be the common phenotypic expression of a large number of different genotypes. Evidence that this is so came from recombination experiments - experiments which, in turn, suggest that it should be possible to make a map of the genome of vaccinia virus. The studies with the white variants, reported in this thesis make a start in this direction.

PART I.

MECHANISMS OF VIRUS VARIATION.

I. MECHANISMS OF VIRUS VARIATION.

Variations in virus properties may be due to hereditary or non-hereditary changes. Inheritable variation may be due to either mutation or some process of transfer of genetic material from one virus strain to another, or from host-cell to virus. Mutations may occur naturally or be artificially induced. If the environment is more favourable for the mutant it will grow from a minority into the majority population as a result of natural selection. The transfer of viruses' genetic material occurs in genetic recombination, which may take place between two viruses infecting a single host cell, or with certain bacteriophages, between the genome of the virus and that of the host bacterium.

Non-hereditary changes can be host-induced. Host-induced modifications cause the properties of some virus types to differ according to the type of host cell in which they are grown.

1. Variation by Mutation.

A mutation is a change in the hereditary material (genome) of the organism or virus, such that one or more of its properties is altered in an observable way.

It is now recognised that most variation in virus properties is caused by random mutation and that virus plasticity results from the variety of genotypes present in large viral populations.

In bacterial viruses variants may arise by a process of mutation occurring during virus replication and mutant clones are found in the virus populations yielded by individual cells. Luria (1951) showed that the distribution of mutant clone sizes among the yields of individual cells is random rather than directed by the host cell.

Very little analytic work has been done to elucidate the processes which give rise to variants in animal viruses. An essential prerequisite for such work is a method of recovering from infected cells clones of virus which are derived from single virus particles. Means must also be available for the easy recognition of genetically determined differences between such clones, and for the indefinite maintenance of virus stocks.

For many years the methods of cultivating and passaging animal viruses have involved the transfer of a virus suspension from one animal or tissue culture to another. It was not known whether the seed material was homogeneous, or how many virus particles were present in the inoculum. During the last decade bacteriological methods of pure culture study have been applied successfully to certain animal viruses, so that methods are now available for the isolation of clones of virus derived from single viral particles. Two principles have been followed, one derived from the colony count methods of bacteriology (Koch 1881)

and the other from limiting dilution assays of bacterial suspensions first recorded by McGrady in 1915 (Eisenhart and Wilson 1943).

Examples of the first principle are the recovery of pocks from the CAM of the chick embryo infected with poxviruses (Burnet 1936, Keogh 1936) and the production of plaques on monolayers of animal cells by polioviruses (Dulbecco and Vogt 1954). In animal virology the limit dilution technique has been principally exploited by Burnet (Burnet and Bull 1943).

Two examples of variation by mutation occurring in animal viruses which have received detail study, are the O-D change of Influenza A virus and the d to d+ mutation of poliovirus.

a. The O-D Change of Influenza A Virus.

The first genetic analysis of variation in animal viruses was the work done on O-D variation. The O-D change of Influenza A virus was first observed in 1943 and described by Burnet and Bull.

On primary isolation from a human patient, influenza A strains were found to agglutinate human or guinea pig red blood cells more readily than fowl cells (Burnet 1942). This virus was said to be in the O or original phase. Paasage in the allantoic cavity or in the amnion at low dilutions led to the appearance of the D or derived phase which readily agglutinated fowl red blood cells. This

change from the O to D phase is associated with a change in affinity for various haemagglutinin inhibitors (Stone 1951) and its position in various receptor gradients (Burnet et al., 1946); and it is also associated with a marked reduction in virulence for man (Burnet and Foley 1940) and emergence of capacity to multiply in the allantois (Burnet and Bull 1943). It has been possible to maintain virus in the O phase through repeated passage in the amnion if each passage is initiated at limit dilution. This shows that prolonged multiplication in the amnion is in itself not an inducer of the change to D phase. D virus appears when a larger amount of virus is inoculated.

The authors concluded from their data that the D phase was due to a spontaneous mutation which when it appeared in the allantoic cavity, rapidly overgrew the original strain.

Unfortunately no estimate of mutation frequency was possible. Intermediates between O and D were observed which could not be easily distinguished from the mixtures of O and D, since the O phase was shown to multiply in the allantois to a limited extent.

b. The d to d+ mutation of Poliovirus.

Dulbecco and Vogt (1958) using the plaque technique (Dulbecco and Vogt 1954) with d lines of poliovirus, have been able to demonstrate the essential character of a mutational change,

namely its randomness. This is the only instance where random occurrence of a mutation during multiplication has been demonstrated with an animal virus.

They showed that the d lines of poliovirus give rise to mutants which have an efficiency of plating under low bicarbonate agar similar to that of wild type virus (d+). These mutants, called "reverse" mutants, provide a good tool for studying the mutability of poliovirus as they could be counted selectively in a large population of d particles. The presence of up to 2×10^5 plaque forming units of d virus in the inoculum did not appreciably affect the titre of the d+ virus. These mutants were detected by the early appearance of plaques on low bicarbonate plates.

The mutants were produced during the growth of d virus in a liquid medium under conditions in which they were not selected for. This was shown by the significant fluctuation in the number of mutants present in sister populations of virus grown in aliquots of one cell population. There was an absence of correlation between total virus assay per aliquot and number of reversion plaques as a consequence of the random occurrence of the mutations during the multiplication of the virus.

2. Genetic Recombination.

Variation in viruses can also occur as a result of genetic transfer. Mixed infection of cells with two viruses which although related, differ in one or more of their known properties, has been shown to yield new types of virus combining characters of both parents. Recombination has been an invaluable tool in bacteriophage genetics, but its exploitation with animal viruses is still in the preliminary stages. Genetic recombination has so far been demonstrated with influenza virus (Burnet 1955), herpes simplex virus (Wildy1955) and vaccinia virus (Fenner and Comben 1958). None of this work has progressed far enough to permit the construction of a genetic map of an animal virus.

3. Host-controlled Variation.

It was an unexpected development when a new type of virus variation was discovered in bacteriophages. This has been called host-induced or host-controlled variation (Novick and Szilard 1951, Luria and Human 1952, Ralston and Krueger 1952, Anderson and Felix 1952, Bertani and Weigle 1953, Luria 1953). Its characteristics are that it is non-hereditary and that it is determined by the host cell in which the virus has been produced. Although such host-induced changes are usually only phenotypic there is evidence of the change eventually being genotypic (Fredericq 1950). The essential

characteristic of host-induced modification is the complete transformation of one form of virus into another upon a single cycle of intracellular growth in a modifying host.

There are at least two examples of what is probably host-induced variation of an animal virus. The first concerns the behaviour of unadapted influenza A virus in mouse brain. After a small inocula of non-neurotropic influenza virus into the mouse brain there is a single cycle of virus production, the virus produced being infective for the allantois but apparently not for the mouse brain in that multiplication there was confined to one cycle (Cairns 1951). The second concerns the behaviour of encephalomyocarditis virus in the mouse brain; a variant of this virus, adapted to form plaques on Sarcoma-180 cells, loses its capacity to form plaques in these cells if passed through a single cycle in mouse brain; this capacity is restored after a single cycle in Krebs 2 carcinoma cells (Sanders and Hoskins 1958).

Summary.

The known mechanisms causing virus variation are:

1. Mutation
2. Genetic recombination
3. Host-induced variation

The best studied examples of mutation among animal viruses have been the O-D change of influenza A virus and the d to d+ mutation

in poliovirus. The d to d+ mutation is the only instance where the essential character of a mutation, namely its randomness, has been demonstrated for animal viruses.

Recombination in animal viruses has been conclusively shown for influenza, herpes simplex, and vaccinia viruses.

Two examples of possible host-induced changes occurring among animal viruses are the alterations in unadapted influenza A and encephalomyocarditis viruses after a single cycle in mouse brain.

PART II.

THE ADAPTATION OF VACCINIA VIRUS TO THE MOUSE BRAIN.

CHAPTER I. HISTORICAL REVIEW.

1. Adaptation to the Rabbit Brain.

The adaptation or increase in virulence of vaccinia virus in the rabbit brain was one of the earliest studied examples of the adaptation of a virus to growth in a new environment and it was the subject of a large number of papers during the period 1920—1940.

Marie (1920) first succeeded in causing illness in rabbits with vaccinia virus by inoculating the virus intracerebrally and transferring the virus from brain to brain successively. Levaditi and his co-workers (1921) developed a strain of vaccinia virus known as "neurovaccinia", which produced regularly, on intracerebral injection into rabbits, encephalitis with paralytic symptoms and death in four to seven days. They regarded "neurovaccinia" as a virus adapted to the central nervous system by passage and considered that it had acquired neurotropic properties. They first adapted it to the brain by alternate brain and testicular passage in the rabbit, later omitting the testicular passages. This method was used by many investigators. Marie, Krumbach, Burnet and Conseil, Condrei, Bachmann and Bilgieri succeeded in producing cerebral symptoms by the intracerebral inoculation in rabbits of stock vaccinia virus. Herzberg claimed to have succeeded in transferring vaccinia virus repeatedly in rabbits by intracerebral inoculation after one passage through rabbit testis. Krumbach claimed that testicular passage was not a necessary condition in obtaining a neurotropic strain, as he obtained

neurotropic properties from a strain which had had cutaneous passage. Levaditi, Lepine and Scheren (1921) decided from their results that a vaccinia strain which had not been passed in rabbits could not become neurotropic to the rabbit.

In the early 1930s a few workers in the United States reported the production of neurovaccinia in rabbits. Thompson (1929) published results of an unsuccessful attempt to grow a vaccinia strain which had been grown in rabbit testis by Noguchi, in rabbit brains. Later he reported (with Buchbinder 1932) successful cultivation of a neurovaccinia from a commercial vaccine without preliminary testicular passage, but he failed to adapt a vaccine from the New York City Health Department. Spooner (1930) adapted a strain derived from the vaccine of the Massachussetts State Health Department. Another worker reported that it had not been possible to adapt the vaccinia virus produced by the Connaught Laboratories, to continued growth in the rabbit brain. Thompson and Buchbinder (1932) investigated the source of vaccinia virus and found that at that time, practically all the virus used in the United States had been derived from the New York City Health Department. It was evident, however, that although all strains of vaccinia virus in the United States had originated from a single stock, different handling had resulted in differences in the ease of adaptation to rabbit brain.

Borrel (1936) suggested that neurovaccinia was really rabbitpox picked up in rabbits' brains during adaptation experiments. Levaditi et al.,(1937) strongly opposed this without any real evidence to support their opposition. They claimed that neurovaccinia was a biological modification of dermal vaccinia. Evidence that not all strains of neurovaccinia could have originated from rabbitpox comes from results of an experiment performed by Miss G. Woodroofe (personal communication). She found that a mutant highly virulent for the rabbit and mouse after intracerebral inoculation was obtained from a mouse brain which had been inoculated with a suspension of a strain of very low virulence. As the original inoculum came from a single pock which had not been passaged in the rabbit, and the properties of the neuropathogenic variant differed from those of the rabbitpox strains used in this department, it could not have occurred as a rabbitpox contaminant. Evidence against neurovaccinia being rabbitpox also comes from experiments performed by Moeljono (1958). He has shown that neurovaccinia is more closely related to vaccinia than to rabbitpox with results obtained from diffusion and precipitation experiments using Ouchterlony plates.

2. Adaptation to the Mouse Brain.

Levaditi and Nicolau (1923) could not obtain a strain neurotropic to the mouse from their neurovaccinia. Rosenau and

Andervont (1931) demonstrated for the first time that mice were susceptible to vaccinia virus inoculated intracerebrally. This, they said, was true only for a specially virulent strain rendered especially active by a particular technique. The strain they used was Armstrong's heat resistant rabbit testicular vaccine (Armstrong) 1929). Like Levaditi and Nicolau they could not infect mice with Levaditi's neurovaccine.

Later both Haagen and Buckup (1935) obtained positive results in mice, the former with a neurovaccinia and the latter with a chorioallantoic culture of vaccinia virus and a neurovaccinia.

Iguchi (1937) used virus from vaccinated humans, which had been passed once through a monkey, four times through rabbit testis and four times through calf skin. Because this material failed to induce brain symptoms it was passed again twice through rabbit testis. After the fourth passage after intracerebral inoculation, it produced brain symptoms in almost all the mice. The survivors were immune to reinfection by the intracerebral route. Repeated brain passage did not further increase the virulence.

Smith, Horgan and Haseeb (1941) produced a neurovaccinia which was of high virulence for the mouse and rabbit after intracerebral inoculation. This was achieved by first inoculating the vaccinia strain intratesticularly in rabbits. They attempted to adapt this mouse-neurovaccinia strain to the sheep brain but they were

unable to do so.

The early workers could not decide whether the differences in adaptibility of various strains were due to a higher concentration of virus in the adaptable material or whether certain properties inherent in all vaccinia virus strains were enhanced by growth in the brain or testis. In the past (1920-1930) it was thought that testicular passage of vaccinia virus was necessary before it could become adapted to the rabbit brain and that only strains which had been grown in the rabbit could be adapted to it. Later (1934) a few workers in the United States found testicular passage unnecessary. For vaccinia virus to be adapted to mice Rosenau and Andervont (1931) thought that only a specially virulent strain such as Armstrong's heat resistant testicular strain, could infect and kill mice.

All these adaptation experiments consisted of the blind passage of unknown quantities of virus material of which the homogeneity was unknown. Therefore in spite of all these studies the process of adaptation of vaccinia virus to the brain is not understood.

In 1936 Keogh showed that neurovaccinia and dermal vaccinia (strains unadapted to the rabbit brain) produced recognisably different pocks on the CAM. It was decided to try to adapt several

of the unadapted strains of vaccinia virus to the mouse brain and to use the chorioallantoic membrane (CAM) as a means of following the processes of adaptation. Virus material as genetically homogeneous as possible was obtained for each vaccinia strain used by using the progeny grown from a single pock (SP). After each mouse brain passage the brain material was inoculated on the CAM so that any changes in pock morphology and any increase in titre of the virus on passage, could be observed.

CHAPTER II. THE GROWTH OF VACCINIA VIRUS IN THE MOUSE BRAIN.

Introduction.

The growth of several strains of vaccinia and two strains of cowpox was followed in the mouse brain, in order to select strains of low virulence for experiments on adaptation.

Experimental Results.

1. Fate of the inoculum after intracerebral inoculation.

Schlesinger (1949) observed that only 3-10% of equine encephalitis virus or bacteriophage could be demonstrated in the brains of mice one hour after inoculation. Cairns (1950) showed that only 2-8% of inoculated bacteriophage could be recovered from the brains of mice five minutes after they had been inoculated intracerebrally, the remainder being rapidly distributed throughout the body of the mouse via the circulation. This loss is unrelated to the volume of the inoculum. My own results with strains of vaccinia and cowpox viruses have been similar; the percentage of virus recovered in the brain varying from 2-13% in different experiments as is shown in Table I.

TABLE I.

Loss of Inoculum after Intracerebral Inoculation.

Virus Strain	Virus titre inoculated in $\log_{10}$ units.	Titre in whole brain 10 mins. after inoculn.	Percentage inoculum remaining in brain
CL-r	5.0	3.5	3
CL-r	4.7	4.0	2
CL	5.3	4.0	5
Led-w	4.7	3.5	7
IHD-w	4.0	2.5	3
HI-w	4.5	3.6	13
WP	5.0	3.6	4
PI	4.8	3.9	9
Nog	4.9	3.5	4
Nel	5.3	4.1	7
RPU	4.7	3.3	4
Gill	5.3	4.1	7
MH	4.6	3.6	10
Led-r	5.2	3.9	5

The sites of multiplication of mouse-virulent strains of vaccinia virus in the mouse brain after intracerebral inoculation have been studied by Dr. C.A.C. Mims of this department (experiments to be published). Using fluorescent antibody he has detected multiplication only in cells lining the cerebro-spinal fluid spaces, that is the meningeal cells, ependymal cells and cells lining the Virchow-Robin spaces. There was no evidence of multiplication of vaccinia virus in the cells of the central nervous system itself. Thus vaccinia virus causes a meningitis rather than an encephalitis

in mice.

Vaccinia virus is quite stable at 37°C when suspended in protective media such as gelatin saline. Its heat inactivation follows that of a second order reaction. In the first two days there is a fall in titre of approximately 1 log. and in the following two days a fall of 0.2 log. To determine whether thermal inactivation of vaccinia virus occurs in mouse brain after intracerebral inoculation, 10^5 pfu of the CL strain were inoculated intracerebrally into fourteen mice and the brains reaped aseptically 5 - 10 minutes later. These were placed in small screw-capped bottles in a 37°C incubator and duplicate brains were removed, ground and titrated at five minute and then daily intervals for seven days. The heat inactivation of the CL strain in gelatin saline and in isolated mouse brain at 37°C is shown in Figure 2.1. In isolated mouse brain the heat inactivation of CL is very slow, and the graph follows a curve similar to that of CL in the brain in vivo after the second day.

2. Growth Curves of the Various Strains.

Mice aged five to six weeks were inoculated intracerebrally with 0.03 ml volumes containing 10^5 pfu of the virus strain, and duplicate brains were removed at the appropriate times, frozen and

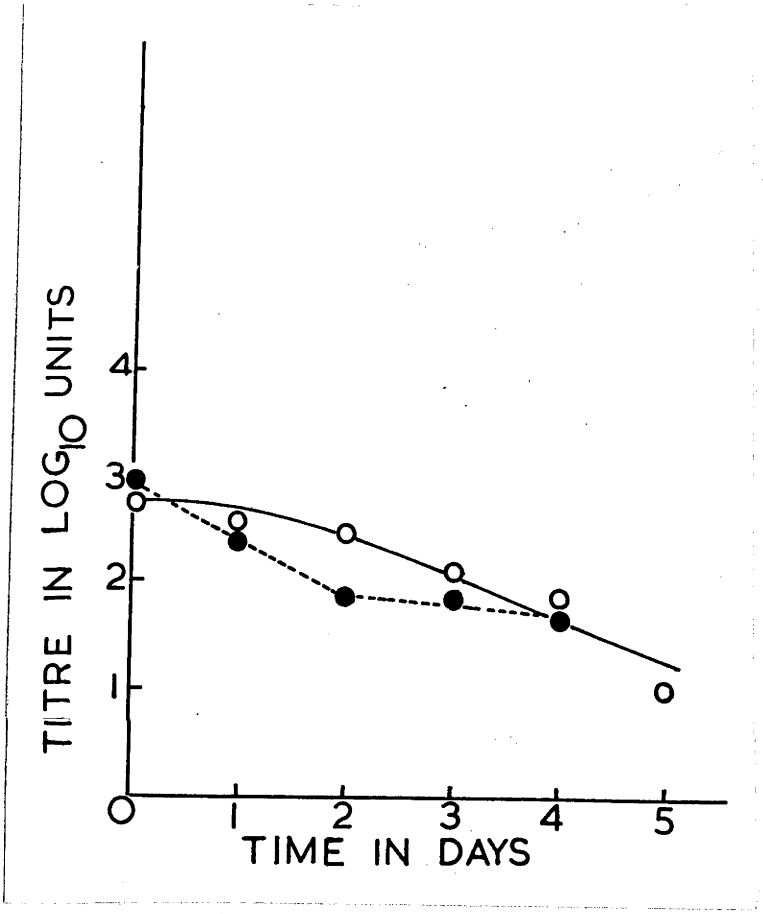


Figure 2.1. The heat inactivation of CL in isolated mouse brain (——) and in gelatin saline (---) both at 37°C.

ground in chilled mortars and pestles, and then suspended in one ml of gelatin saline per brain. Their virus content was titrated according to the pock counting method of Beveridge and Burnet (1946) and recorded in $\log_{10}$ units of whole mouse brain. Details of the method are given in the appendix.

Curves were constructed on the basis of titrations carried out 5 minutes, 8 hours and daily for seven days after intracerebral inoculation. Figures 2.2 - 2.9 show the growth curves of the various strains.

Discussion.

1. Differences in behaviour of Different Strains.

As a result of this experiment it is possible to divide the strains into three groups based on degrees of virulence for the mouse host. Five strains were of high virulence (CL-r, RPU, IHD, Nog and HI) causing the death of all mice in three to five days, five strains were of intermediate virulence (WP, RPU-w, Nel, HI-w and IHD-w) killing 20% or more of the mice, and nine strains were of low virulence (Gill, MH, Led-r, PI, CPA-w, CPLB-w, CL and 7N) killing less than 10% of the mice.

The early workers (Buddingh 1936) classified the vaccinia

(The growth curves of IHD, HI and 7N were obtained from Miss G. Woodroofe
(of this Department.

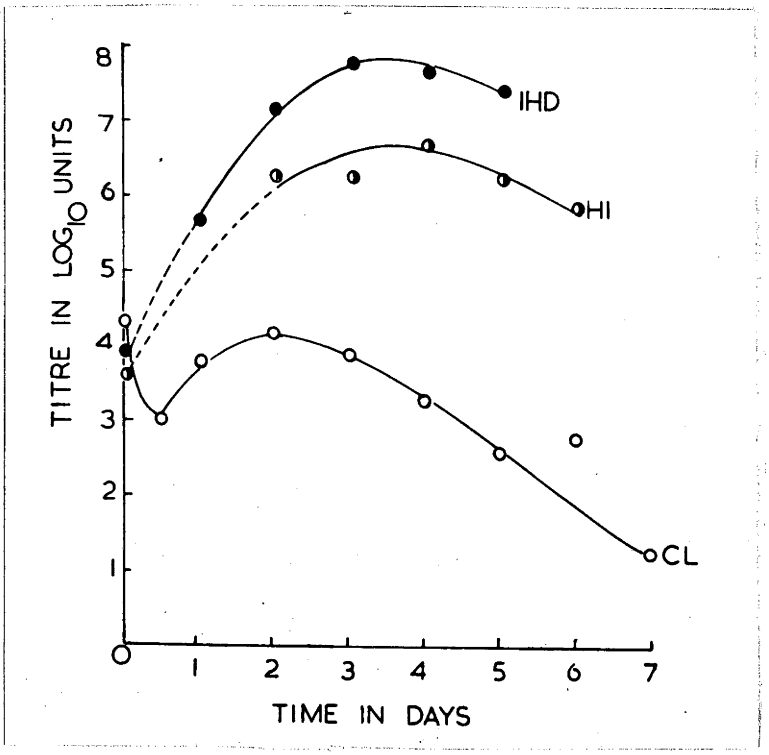


Figure 2.2a. The growth of IHD and HI as compared with a strain of very low virulence(CL), in mouse brain.

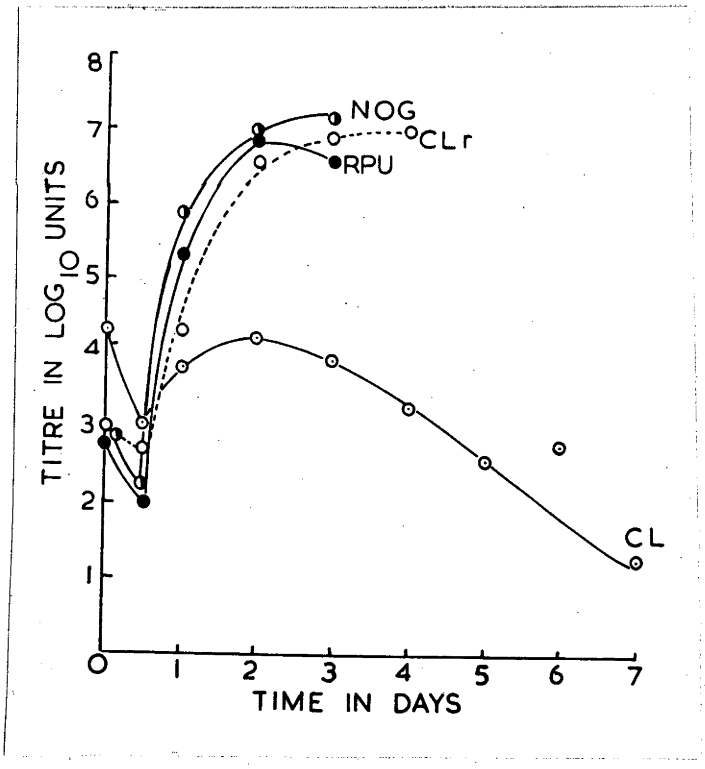
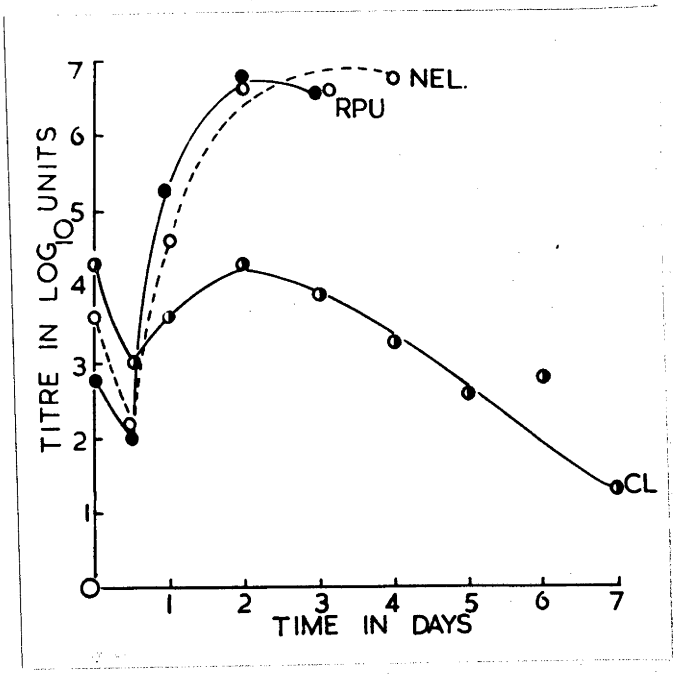
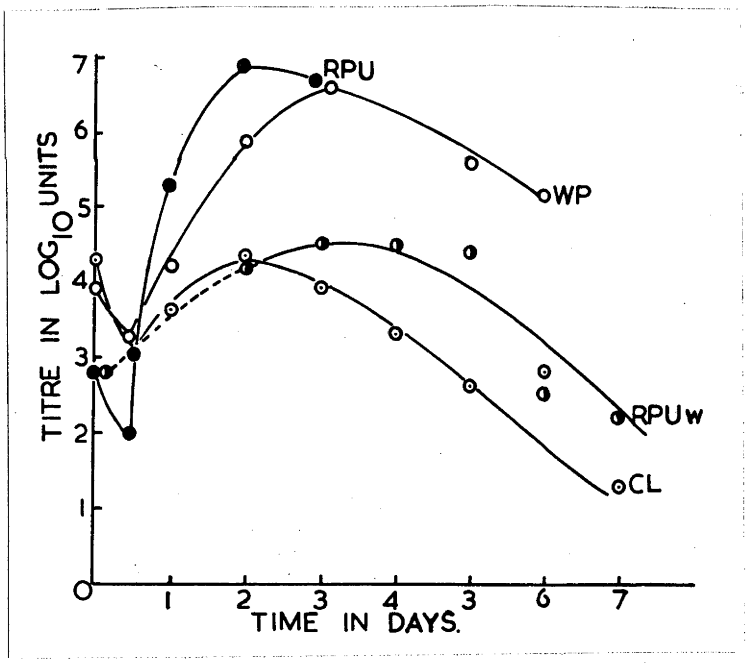


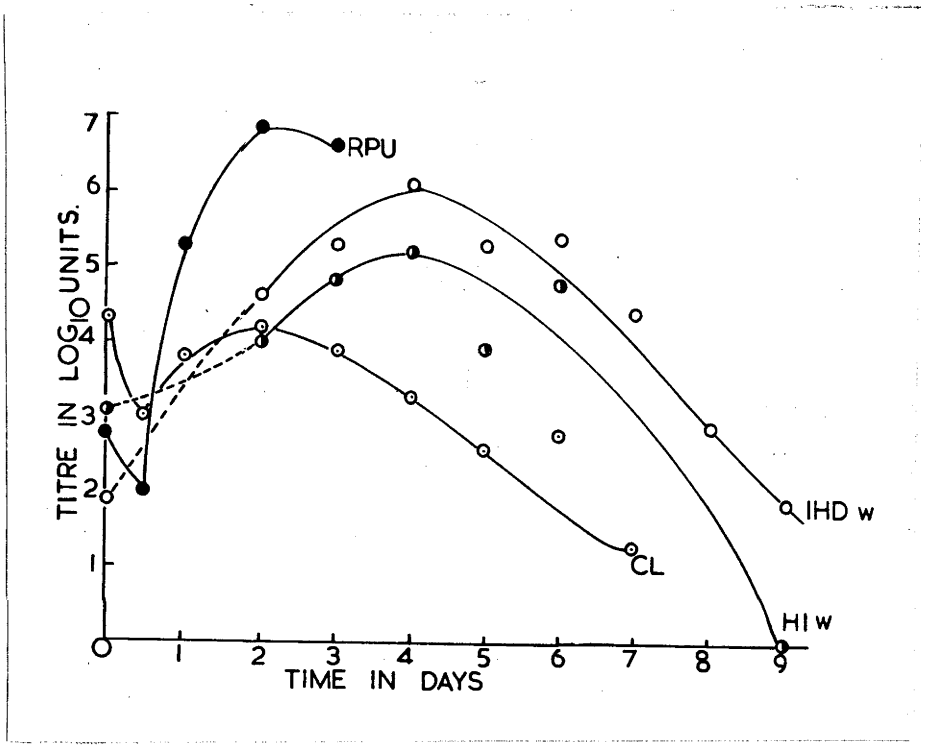
Figure 2.2b. The growth of Nog, Cl-r and RPU in mouse brain.



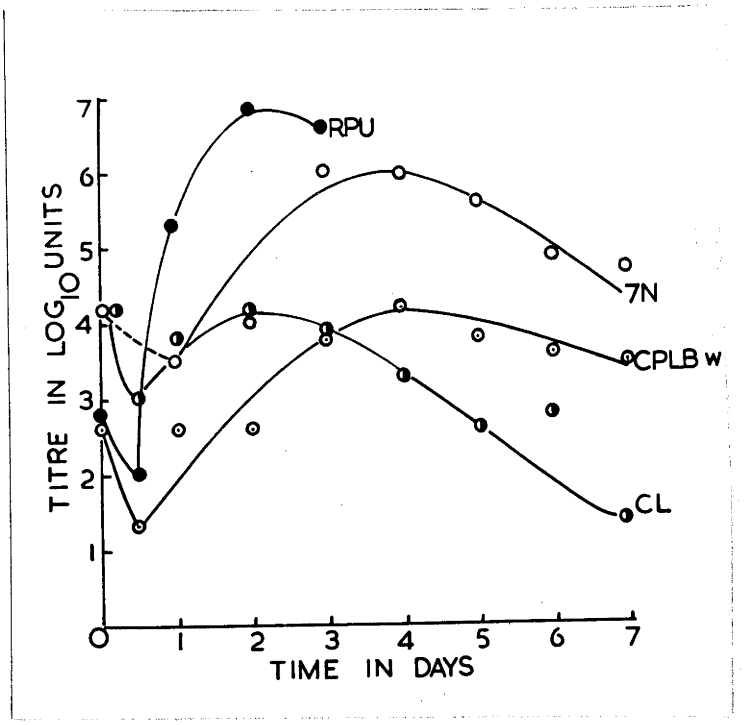
GROUP II. Figure 2.3. The growth of Nel compared with a strain of high and a strain of low virulence in the mouse brain.



GROUP II. Figure 2.4. The growth of WP and RPU-w in mouse brain.



GROUP II. Figure 2.5. The growth of IHD-w and HI-w compared with strains of high and low virulence in mouse brain.



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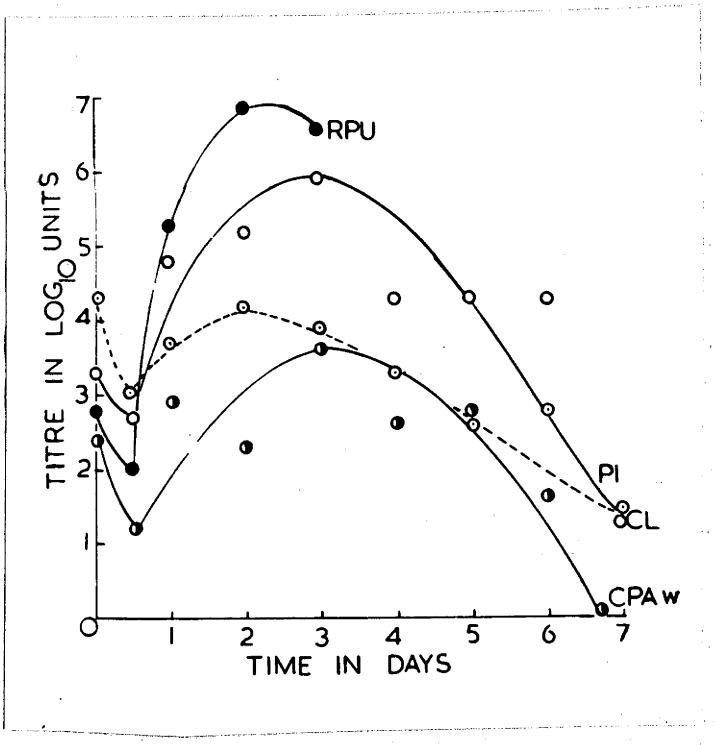
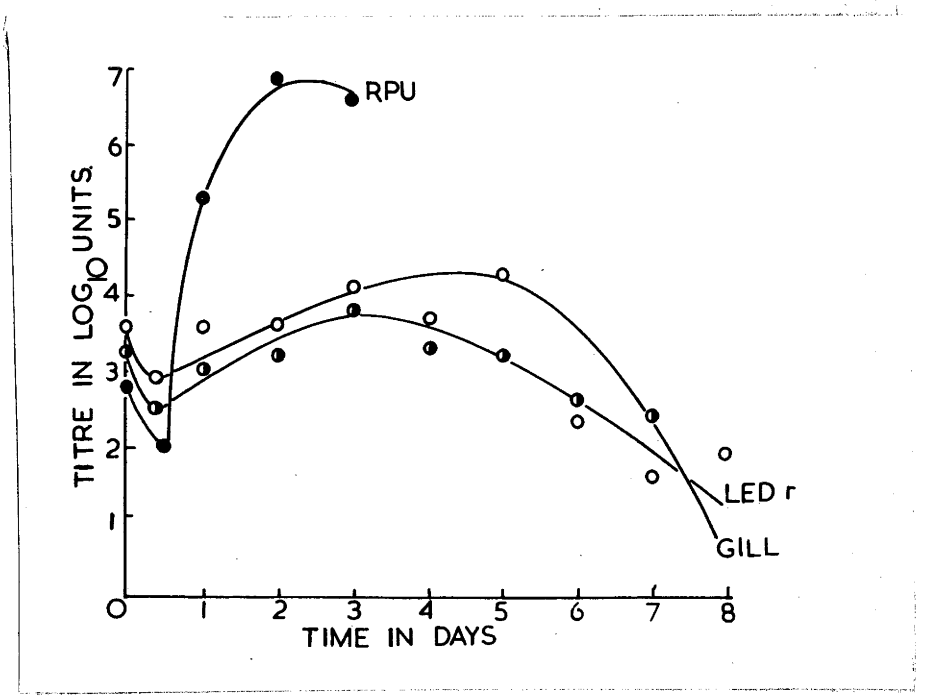


Figures 2.6 - 2.7. The growth of some of the low virulent strains compared with a strain of high virulence in mouse brain.



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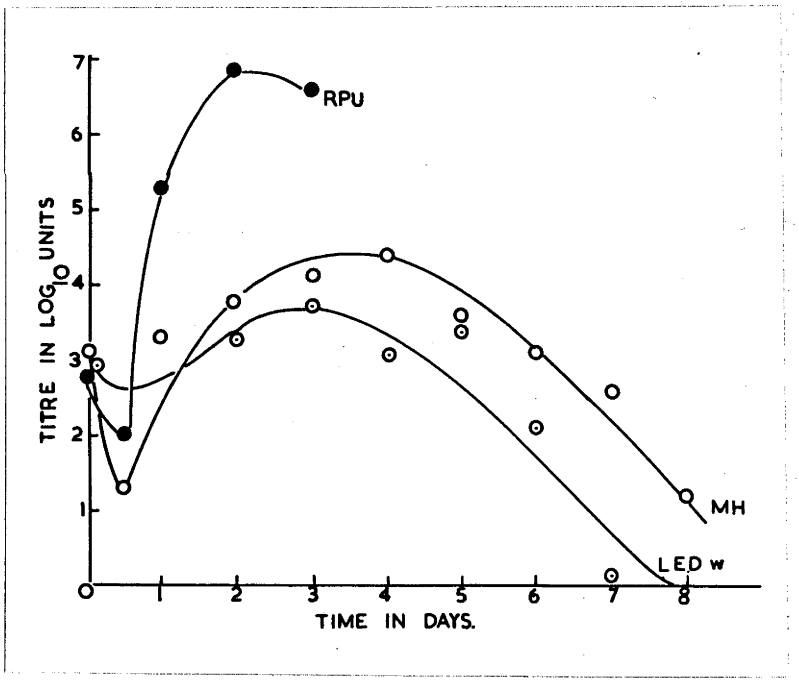


Figures 2.8 - 2.9. The growth of several of the strains of low virulence compared with that of a strain of high virulence, in mouse brain.

TABLE II.

Some of the Properties of Strains of Vaccinia and Cowpox Viruses.

	Cl.r	IHD	HI	RPU	Nog	IHDw	HIw	RPUw	Nel	WP	CL	Gill	MH	Ledr	Ledw	7n	PI	CPAw	CPLBw
Group	N	N	N	N	N	D	D	D	D	D	D	D	D	D	D	D	N	D	D
Pock type	r	r	r	r	w	w	w	w	w	w	w	w	w	r	w	w	r	w	w
Mouse virulence i/c	+++	+++	+++	+++	+++	++	++	+	++	++	-	-	+	+	-	+	+	-	-
Rabbit virulence i/c	+++	+++	+++	+++	+++	+++	+	-	-	++	-	-	-	-	-	-	++	-	-
Type of lesion in rabbit skin	h	h	h	h	h	n	n	n	n	n	n	n	n	n	n	n	h	n	n

i/c = Intracerebral inoculation
N = Neurovaccinia
D = Dermal
r = Red pock
w = White pock
+++ = High virulence
- = Avirulent
h = Haemorrhagic lesion
n = Nodule

strains into two groups, namely dermal strains and neurovaccinia strains. Strains which had been passed repeatedly in the rabbit skin were called dermal strains to distinguish them from the "neurovaccinia" produced by serial passage of vaccinia in the rabbit brain. Dermal strains produced nodular lesions without necrosis on intradermal inoculation in rabbit skin, whereas neurovaccinia strains produced much larger, flat lesions with central necrosis and haemorrhage. Other properties of these strains have been listed by Fenner (1958) and the relevant ones are shown in Table II.

Group I This group contains the strains of high virulence for mice. When inoculated into rabbit skin all these strains produced lesions with haemorrhage and necrosis which classified them as neurovaccinia. Except for Nog which produced a white pock, all these strains produced a haemorrhagic pock on the CAM which is also characteristic of neurovaccinia strains. They were all highly virulent for rabbits after intracerebral inoculation.

Group II This consists of all strains of intermediate virulence for mice. They all produced nodules in the rabbit skin after intradermal inoculation and white pocks after inoculation on the CAM, both of which are characteristic of dermal vaccinia. Strains in this group varied in their virulence for rabbits after intracerebral inoculation.

Group III The strains of low virulence belong to this group. With the exception of PI, all the strains produced nodules in the rabbit skin and therefore belong to the dermal vaccinia group. PI is a direct descendant of Levaditi's and Nicolau's (1923) neurovaccinia, and as well as producing haemorrhagic lesions on the rabbits' back was virulent for rabbits after intracerebral inoculation. It is therefore classed as a neurovaccinia strain. The other strains of this group were a-virulent for rabbits after intracerebral inoculation. PI and Led-r form haemorrhagic pocks on the CAM and in this respect behaved like neurovaccinia, but Led-r was similar to the dermal vaccinia strains in all its other properties. Although PI behaved as a neurovaccinia it was of low virulence for mice and for this reason it belongs to Group III.

2. Analysis of Growth in the Mouse Brain.

All the strains showed some evidence of multiplication in the mouse brain. After the initial drop in titre of approximately 90%, there was a short lag phase then a further drop in titre for eight to twelve hours.

The decline in infectivity between five minutes and eight to twelve hours is similar to that observed in the growth of vaccinia virus in the CAM (Briody and Stannard 1951, Anderson 1954 and Metcalf 1955) and its growth in pieces of a rabbit's ear incubated in a

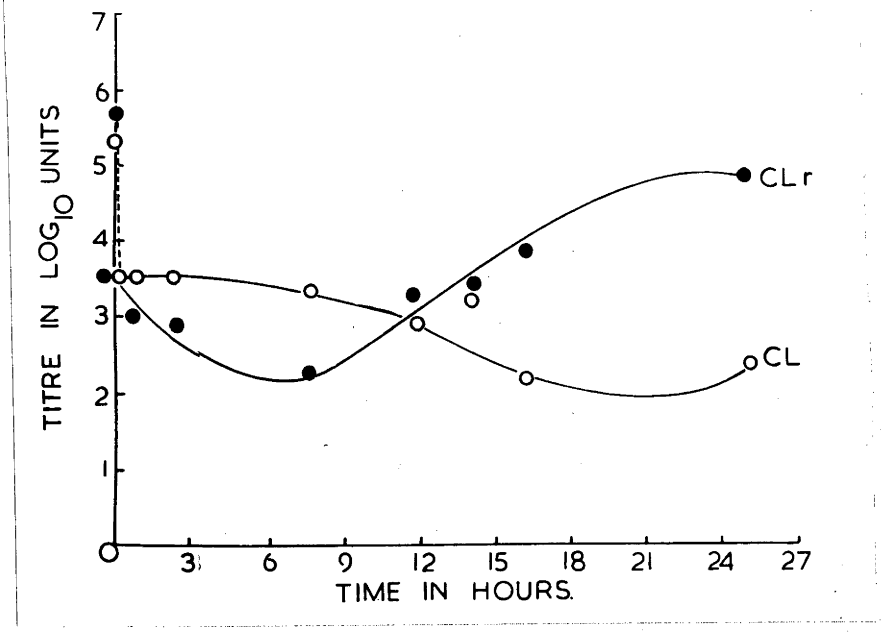


Figure 2.10. A comparison of the eclipse phases in mouse brain, of a very low virulent strain(CL) with a highly virulent strain (CL-r).

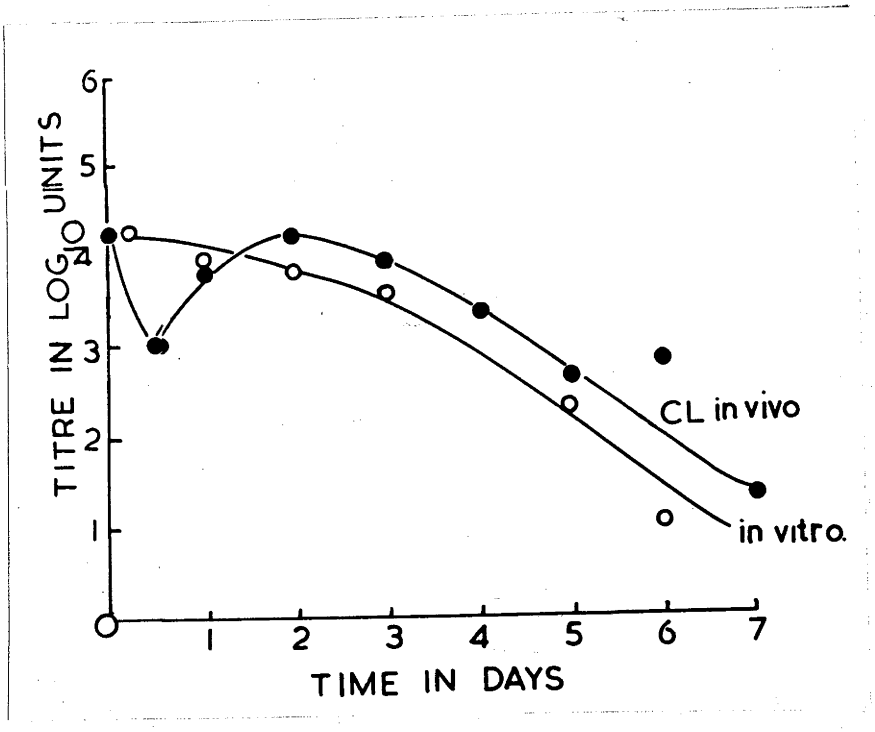


Figure 2.11. The growth of CL in mouse brain *in vivo* compared with CL heat inactivation in mouse brain *in vitro*.

nutrient medium after intradermal inoculation (Crawford and Sanders 1952). This disappearance of an inoculum in an experimental virus host complex is well documented for many systems (Henle 1949, Ginsberg and Horsefall 1951, Doermann 1952 and Rubin, Baluda and Hotchin 1955) and was observed for all the strains of vaccinia virus used in these experiments.

To detect any differences which may occur between strains of high and low virulence during the eclipse phase, the titres of CL, a strain of low virulence, and CL-r a virulent strain, were followed more closely by removing brains at shorter time intervals. The curves obtained are shown in Figure 2.10 and show the longer eclipse phase of the attenuated virus.

No eclipse phase was observed with vaccinia virus in the brains incubated in vitro. In this case the titre decreased slowly over a period of days. The infectivity titration of CL in vivo followed that of CL in vitro very closely after the second day of inoculation, but not for the first two days. It was in the first two days that CL showed evidence of multiplication, but compared with that of strains belonging to Groups I and II it was very slight. The brains in vitro however, quickly degenerated and autolysis of the cells occurred, so that the condition of the brain in vitro was very different from that in vivo. For this reason the fact that the two curves are similar after the second day may be coincidental.

Figure 2.1 shows the growth of CL in the mouse brain *in vivo* together with the decrease of CL infectivity in the brain *in vitro*.

The rate of multiplication and the highest titre reached were also closely correlated with the virulence of the different strains as is seen in the Figures 2.2 - 2.9.

The growth rate of vaccinia virus in the mouse brain has also been recorded by Minton, Officer, Hitchins and Thompson (1953) using the Williamsport strain. Multiplication did not begin for eight to twelve hours after inoculation of the virus, and then growth proceeded at a regular rate until the maximum titre was reached. The virus titre was measured by the ID_{50} intradermally in rabbits, and the highest titre reached was found to be approximately the same regardless of the quantity of virus injected.

Summary.

The results of the growth of several strains of vaccinia and two strains of cowpox virus in the mouse brain, divide these strains into three groups based on virulence for the mouse host.

The early workers divided the strains into two groups according to the type of lesion they produced in the rabbit skin. The neurovaccinia strains produced a haemorrhagic lesion and the dermal strains produced a nodular type of lesion. According to this classification all the strains of Group I are neurovaccinia strains,

all those belonging to Group II are dermal strains and except for PI, all the strains in Group III are dermal strains too. The strains in Group I are all virulent for the rabbit and with the exception of Nog all produce red pocks on the CAM. All the strains in Group II form white pocks but they vary in virulence for the rabbit. With the exception of PI and Led-r which form red pocks, the other strains of Group III produce white pocks. They are all a-virulent for rabbits with the exception of PI which is the only neurovaccinia not virulent for mice.

In the mouse brain the virulent strains have a shorter eclipse phase, a higher multiplication rate, and reach a higher titre than the strains of low virulence.

CHAPTER III. THE ADAPTATION OF VACCINIA VIRUS TO THE MOUSE BRAIN.

Introduction.

Several of the strains of vaccinia virus of low and intermediate virulence described in Chapter II were selected for experiments on adaptation to the mouse brain. These were designed to allow a systematic examination of the process where strains which although initially not highly virulent for mice after intracerebral inoculation, altered on serial passage so that they eventually produced fatal infection.

Experimental Results.

1. Attempts fo adapt several strains of low and intermediate virulence to the mouse brain.

The strains used were:

RPU-w
Led-w
IHD-w
Led-r
HI-w
Gill
MH
PI
CPA-w
CPLB-w

The origins of these strains are described in the appendix. Doses of 10^5 pfu of each of these strains which are of intermediate or low mouse virulence, were inoculated into groups of mice. A brain was removed from each group on the third or fourth day after

inoculation, when the virus titre was highest (see Chapter II of this part). From these brain suspensions further passages were made every three or four days in groups of mice, and were continued for five passages. Brains were removed from any sick or dead mice after the third day and this material was also passaged. In order to avoid the deaths which occurred in a proportion of mice immediately after the intracerebral inoculation of very concentrated brain suspensions, 10% suspensions were used for passages.

The results of serial passage were assessed after the fifth passage. If the brains removed on the fourth day at this time contained no virus when tested on the CAM, brain suspensions from earlier passages, which had been stored at -65°C, were examined to see when the serial passage had failed. These results together with the number of deaths which occurred during passage are shown in Table III.

RPU-w and IHD-w were the only strains to increase in titre (and virulence) on serial passage. The titre of PI remained at a high level in all five passages but was never lethal. The titres of the other strains decreased during passage and most strains did not survive the second passage.

These experiments were undertaken in the hope that changes in mouse virulence might be accompanied by changes in pock morphology

TABLE III.

Serial Mouse Brain Passage (brains being removed on 4th day after inoculation) of several strains of Vaccinia Virus and two strains of Cowpox Virus.

Passage	RPU-w	IHD-w	HI-w	PI	Led-r	Led-w	MH	Gill	CPA-w	CPLB-w
1. a.	0/5	3/4	3/10	1/5	4/5	0/5	1/5	0/5	0/5	0/5
b.	4.6	6.1	5.2	5.3	3.9	3.3	3.9	0.0	2.6	3.0
2. a.	0/5	4/4	1/4	0/5	0/5	0/5	0/5		0/5	0/5
b.	4.7	6.0	5.6	-	2.3	0.0	0.0		0.0	0.0
3. a.	2/6	4/4	1/4	0/5	0/5					
b.	5.0	6.0	4.7	5.9	0.0					
4. a.	3/3	2/2	1/5	0/5						
b.	5.6	7.3	3.9	5.8						
5. a.	4/9	2/2	0/5	0/5						
b.	6.6	6.6	3.9	5.3						

a = Number of deaths which occurred between the third and fourteenth day after inoculation.

b = Titre on fourth day of whole brain in $\log_{10}$ units.

on the CAM on which neurovaccinia strains had already been shown to differ markedly from dermal vaccinia (Keogh 1936). However, the observed increase in virulence of RPU-w and IHD-w was not accompanied by any changes in pock morphology on the CAM, so that the process of adaptation could not be followed in this way.

Two pocks of RPU-w and IHD-w were isolated from the fifth passage material and to obtain sufficient virus to inoculate doses of 10^5 pfu into the brains of mice, confluent membranes were prepared from the SP suspensions. Their mouse virulence was compared with 10^5 pfu of the stock material and 10^5 pfu of several SPs isolated from the stock virus. The results are shown in Table IV.

The virus produced by the two SPs isolated from passage five of both RPU-w and IHD-w, was more virulent than the original stock virus and the virus derived from individual SPs of stock virus, as determined by the number of deaths and the days on which these deaths occurred.

Stock RPU-w was not homogeneous with respect to mouse virulence. The percentage of deaths caused by the different clones derived from this stock ranged from nil to 80%. The variation in virulence of clones derived from stock IHD-w was less, but some killed all the mice inoculated.

TABLE IV

The comparison of virulence of RPU-w and IHD-w virus isolated from the fifth mouse brain passage, with that of the original RPU-w and IHD-w material and also with that of the progeny of particles isolated from the original RPU-w and IHD-w material.

Virus Strain	No. of deaths / Total No. of mice inoculated	Days on which death occurred
SP1 RPU-w) from mouse brain material of passage 5	7/7	2 on 3rd day 3 on 4th day 2 on 5th day
SP2 RPU-w) from mouse brain material of passage 5	8/8	2 on 4th day 4 on 5th day 2 on 6th day
Stock RPU-w	1/7	1 on 4th day
S.P. 1 - 10 isolated from the stock material RPU-w	0/5	3 on 3rd day 18 on 4th day 55 on 5th day 10 on 6th day 7 on 7th day 7 on 8th day 4 on 9th day 4 on 12th day 2 on 13th day
11 - 16 isolated from the stock material RPU-w	1/5	
17 isolated from the stock material RPU-w	2/5	
18 - 19 isolated from the stock material RPU-w	3/5	
20 - 22 isolated from the stock material RPU-w	4/5	
SP1 IHD-w) from mouse brain material of passage 5	9/9	3 on 3rd day 3 on 4th day 2 on 5th day 1 on 6th day
SP2 IHD-w) from mouse brain material of passage 5	7/7	4 on 3rd day 3 on 4th day
Stock IHD-w	5/7	3 on 5th day 2 on 7th day
SP 1 isolated from the stock material IHD-w	3/5	5th - 8th day
2 - 7 isolated from the stock material IHD-w	4/5	
8 - 10 isolated from the stock material IHD-w	5/5	

These results can be interpreted as meaning either that the virulence of RPU-w and IHD-w had increased on passage, or that virulent mutants already present in the original material were selected on passage. The results fail to obtain any correlation between mouse virulence and pock morphology.

2. Adaptation of CL, a strain of very low virulence, to the mouse brain.

a). The occurrence of a red-pock forming mutant on brain passage.

Other than the white variants of neurovaccinia (to be dealt with in Part III) only one pock variant was seen in experiments on the growth of vaccinia strains in mouse brain. This occurred in an experiment carried out by Miss G. Woodroofe. She found that there were red pocks as well as the usual white pocks on CAMs inoculated with a mouse brain which had been reaped on the fifth day after intracerebral inoculation of 10^5 pfu of CL. When this mouse brain was diluted 1/120 in gelatin saline an average of two red pocks and twenty-three white pocks per CAM was estimated from pock counts of ten membranes. These red pocks were designated CL-r. This brain suspension was passed intracerebrally into mice and continuous brain passages were made every three or four days. By the third passage all the pocks seen on the CAMs were red.

To determine whether the red mutant had increased in virulence during these passages, five red single pocks were isolated from the original brain suspension and five were isolated from the fifth passage brain suspension, in which all pocks were of the red type. These ten SPs were inoculated intracerebrally into ten groups of six mice and the survival times of the mice were recorded. No significant differences in the survival times were observed for these ten mutants as that the virulence of the mutant had not increased on passage. The virulence of the mutant (CL-r) for mice was similar to that of neurovaccinia (see Chapter II of this part). After intracerebral inoculation of 10^5 pfu of the mutant all the mice died with death times comparable with those of neurovaccinia strains.

To determine whether the original strain CL interfered with the growth of its mutant CL-r in the mouse brain, various proportions of CL and CL-r were inoculated together intracerebrally into groups of mice and two brains per Group were reaped on the fourth day and titrated on the CAM. The number of deaths and the days on which these occurred were recorded. The results are described in Table V.

Analysis of these results showed that CL interfered with the

TABLE V.

The effect of CL on the growth and virulence of CL-r when both are inoculated together into the mouse brain.

Titre of virus inoculated.	no.deaths/total no. inoculated.	Days deaths occurred.	Titres of brains on 4 day.
10^5 pfu CL	0/8		1.8w
10^5 pfu CL + 1 pfu CL-r	0/8		1.8w 0.0r
1 pfu CL-r	3/8	8,8,9,	0.0r
10^4 pfu CL + 10 pfu CL-r	1/8	4,	1.6w 1.3r
10 pfu CL-r	7/7	6677789,	4.3r
10^3 pfu CL + 10^2 pfu CL-r	2/8	4,5,	1.6w 4.7r
10^2 pfu CL-r	8/8	66777778,	5.1r
10^2 pfu CL + 10^3 pfu CL-r	8/8	45556710,10,	0.0w 6.3r
10^3 pfu CL-r	7/7	5566666,	6.3r

growth of CL-r when they were inoculated together into the mouse brain, and as a result of this there was a decrease in the number of deaths expected, for example, when 10 pfu of CL-r were inoculated its titre on the fourth day was 4.3 and all the mice died, but when 10,000 pfu of CL were inoculated with 10 pfu of CL-r only one out of the eight mice inoculated died and the CL-r titre on the fourth day was only 1.3. Even the presence of 1 pfu of CL to 10 pfu of CL-r caused a lag in the death rate of the mice, although none of the CL strain was seen in the mice brain material reaped on the fourth day and titrated on the CAM.

The Table also shows that as the number of pfu of CL-r inoculated into mice brains was increased, the mice died earlier.

This example of the adaptation of CL to the mouse brain can be interpreted as due to a single-step mutation to high virulence. This mutant did not increase further in virulence during passage, but because of its faster multiplication rate it outgrew the original strain (see Chapter II of this section).

b). Attempts to repeat the adaptation of CL to mouse brain.

Growth of the CL strain in the mousebrain was repeated seven times to see if the red pock forming mutant could be obtained again. These experiments were unsuccessful.

High concentrations of CL were obtained by genetron extraction (Gessler et al., 1956) of the virus from confluent CAMs and concentration of the extracted virus by ultracentrifugation. Doses containing approximately 10^8 pfu of CL were inoculated intracerebrally into forty mice and the brains were reaped, ten each on the first, second, third and fourth days. These brains were ground separately and inoculated onto CAMs to see whether any red pocks could be seen. This experiment was repeated three times but no red pocks were seen. Some of the mice were sick on the first day after the inoculation of this very high concentration of virus, but all subsequently recovered. Alternate mouse brain (with brains being reaped after three or four days virus growth) and CAM passages for twelve passages also failed to yield any red mutants or adapt the strain to the mouse brain.

Discussion.

Fenner (1958) has shown the great variability in the characters of strains of vaccinia virus. The results of these experiments show that there are also differences in the adaptability of the different strains to the mouse brain.

1. Adaptation of RPU-w and IHD-w to the mouse brain.

Serial passage of eight strains of vaccinia virus and two strains of cowpox resulted in an increase in titre and virulence of

two strains, RPU-w and IHD-w. Except for PI, whose titre remained constant, the other strains decreased in titre during passage and most strains did not survive the second passage.

Unfortunately no changes in pock morphology were observed during the increase in virulence of RPU-w and IHD-w, therefore the CAM could not be used to observe the processes involved in the adaptation of these strains.

2. Adaptation of CL to the mouse brain.

Miss G. Woodroofe isolated a virulent mutant from a mouse brain which had been inoculated with CL. This mutant was detected on the CAM as it formed red pocks whereas the parent strain formed white pocks. After three mouse brain passages the brain material, when titrated on the CAM, contained only the red pock forming mutant. This did not increase further in virulence on passage. The original mutant was fully virulent for mice and therefore like the neurovaccinia strains described in Chapter II of this section. The proportion of the mutant increased on passage as a result of selection so that the number of deaths increased and the survival time was shortened.

This mutation to full virulence proved to be a rare one however, for although the experiment was repeated ten times with even higher conentrations of CL, it was not detected again.

These experiments have shown that there are differences in the adaptability and in the processes of adaptation to the mouse brain of the different strains. The adaptation of CL probably occurred as a result of a one-step mutation from non-virulence to high virulence. Clones of intermediate virulence were isolated from the stock RPU-w and IHD-w, which suggested that the observed adaptation could have occurred by progressive selection.

Summary.

The vaccinia strains studied varied in their ability to become adapted to the mouse brain. Two vaccinia strains out of eight strains of vaccinia and two strains of cowpox were adapted after five serial passages.

The CAM could not be used to observe the processes of adaptation of RPU-w and IHD-w as they did not involve a change in pock morphology. The adaptation of CL to the mouse brain by Miss G. Woodroofe, has been followed clearly on the CAM because of the different pock morphology of the mutant, and occurred as a result of a single step mutation to high virulence of a CL particle, followed by selection of the nutant in the mouse brain. The adaptation of RPU-w and IHD-w to the mouse brain probably occurred by way of progressive selection.

PART III.

THE WHITE VARIANTS OF RABBITPOX VIRUS.

Introduction.

Downie and Haddock (1952, 1953) isolated a white variant which appeared during the propagation of a strain of cowpox on the CAM. The pocks produced by the parent virus were haemorrhagic ulcers about two millimeters in diameter whereas the variant produced non-haemorrhagic white pocks. When isolated as a pure strain, the white variant was found to differ from the parent not only in pock morphology, but also in other characters such as virulence for mice and rabbits. They estimated that the frequency of occurrence of white variants was 1/40 to 1/300. Fenner (1958) found that white variants occur with somewhat similar frequencies among all those mammalian pox viruses which produce red pocks on the CAM.

In view of the exceptionally high frequency of white variants it seemed possible either that the white pock was the common phenotypic expression of a large number of different genotypes, or that it represented a host-induced change similar to that described for bacterial viruses (Novick and Szilard 1951, Luria and Human 1952 etc.). The following experiments were performed to test these hypotheses.

The white variants examined were isolated from the red pock producer, RPU. This strain was chosen because it was one of the parents in the recombination experiments of Fenner and Comben (1958)

and it was important to compare the range of characters of a number of white variants of RPU with those of the recombinants, the great majority of which produced white pocks.

Experimental Results.

1. The reliability of estimating the frequency of occurrence of the white variants.

It was first necessary to determine the reliability of identification of the white variants of RPU on membranes where the majority of pocks were of the RPU type. The following experiment, the details of which were kindly supplied by Professor Fenner, was carried out.

A clone of RPU was selected which gave a relatively low frequency of white variants (approximately 1w per 800r when 3,000 pocks were counted). Mixtures of known concentrations were then made with this clone and preparations of the white variant described previously by Fenner (Fenner 1958). Counts were made two days after the inoculation of the various concentrations on the CAMs. The results are shown in Table VI.

These results show that the ratio of white to red pocks in counts made at appropriate dilutions corresponds approximately to the known input ratio. The figure is more accurate with the higher ratios of white to red pocks and the lower pock counts. It is not reliable when the membrane is covered with red pocks.

TABLE VI

Test of the reliability of identification of white variants (RPU-w) on membranes containing a majority of reds (RPU).

Concentration of r/w mixed.	Dilution.	Counts obtained.	r/w obtained.	
1,000/1w	10^{-3}	500r 1w 500r 3w 200r 1w	1,200/5	240/1
	10^{-4}	29r 0w 7r 0w	36/0	
100/1	10^{-3}	34r 1w 60r 0w	94r/1	100/1
10/1	10^{-3}	150r 12w 128r 12w semicon r 28w	550/52	10/1
	10^{-4}	44r 4w 30r 5w	74r/9	8/1

2. The occurrence of white variants from RPU when grown in different hosts.

All the experiments with white variants reported so far (Downie and Haddock 1952, van Tongeren 1952 and Fenner 1958) were carried out with viruses grown on the CAM. In view of the high frequency with which these variants occurred it seemed possible that they represented some sort of host-induced change. To examine this possibility clones of RPU were obtained from several different hosts, and tested on the CAM.

The poxviruses grow from small inocula both in the rabbit skin and mouse brain (Fenner 1958) and also produce plaques in chick embryo fibroblast monolayers (Noyes 1953). RPU is highly virulent for the mouse and the rabbit, the infectivity endpoints in both being similar to that found on the CAM (Fenner 1958). I have found that RPU also produces plaques in chick fibroblast monolayers with about the same efficiency of plating as pocks on the CAM.

The proportions of white pock producing particles obtained from a plaque, from the skin nodule on a rabbit and from the brains of mice, each infected with a single infective particle of RPU (this is obtained by using the limit dilution technique described in the appendix) were compared with each other and with the proportion of white pocks produced from the progeny of several single infective particles grown on the CAM. Each sample was inoculated at the

appropiate dilution onto 20 - 40 CAMs and the number of white and red pocks were counted two days later. The results are shown in Table VII.

TABLE VII.

The percentage of white variants in clones of RPU produced from single infective particles in several different hosts.

Origin.	Sample No.	Total w/r.	%w.	Average %.
Pocks + on CAM.	1	1/183	0.6	2
	2	6/363	1.7	
	3	1/223	0.5	
	4	4/235	1.8	
	5	4/367	1.1	
	6	5/790	0.6	
	7	12/87	14.0	
	8	3/379	1.0	
Nodules in Rabbit skin.	1	11/475	2.0	2
	2	24/1005	2.0	
Mouse Brain	1	4/400	1.0	1
	2	12/1406	1.0	
Plaque on chick embryo fibroblasts	1	3/680	0.5	0.5

+ Data supplied by Professor Fenner.

With the larger series of pocks from the CAM it is apparent that there was a good deal of variability in the proportion of white to red pocks. This result is compatable with, but not

sufficiently extensive or accurate to demonstrate the existence of fluctuation. The overall white percentage was about two percent.

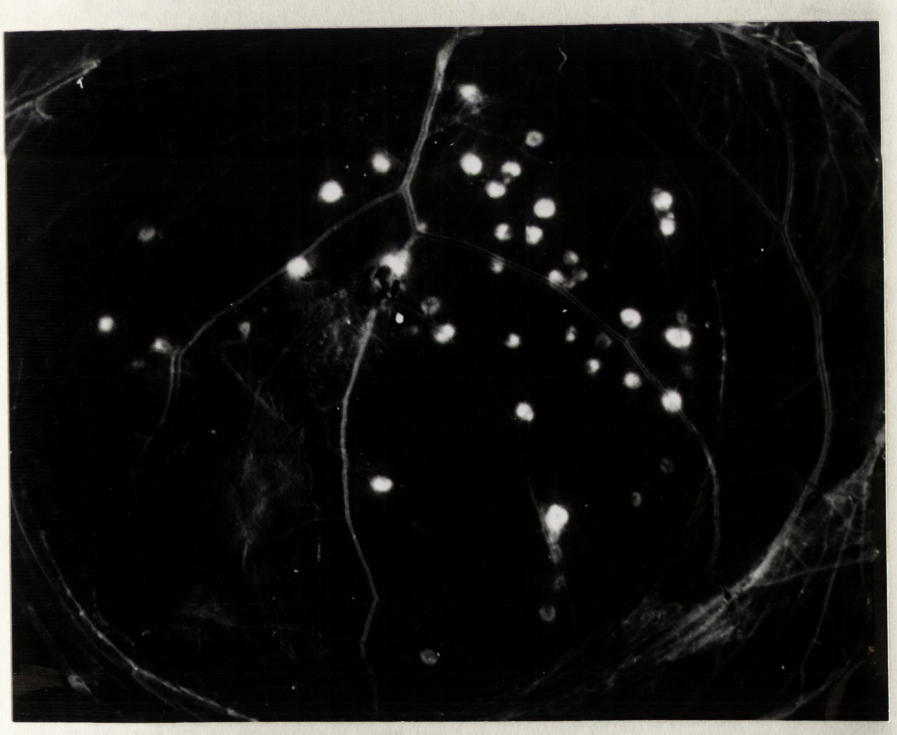
Although only a few clones produced in other host systems were examined, the ratios of white to red pocks in each did not greatly differ from those obtained with several pocks grown on the CAM. Host factors operating during the time of development of the clones of RPU do not seem to affect the likelihood of production of particles which will produce white pocks.

3. Isolation of the white variants.

Because of the high frequency of occurrense of white variants it was possible that although phenotypically similar they may be genotypically different. A number of them were therefore isolated and purified so that their properties could be examined and any differences detected. The white variants were selected from the progeny of a single particle of RPU grown on the CAM.

This single RPU pock was removed from the CAM with sterile scissors and forceps and placed in a small glass grinder at -4° C until it had frozen. The pock was then ground until it had thawed and suspended in one ml of gelatin saline. After sonication it was inoculated onto a large number of CAMs at a dilution which would give approximately 100 RPU pocks and one to two white pocks per membrane.

Figure 3.1. w1 of Group I.



Fiigure 3.2. w7 of Group II.

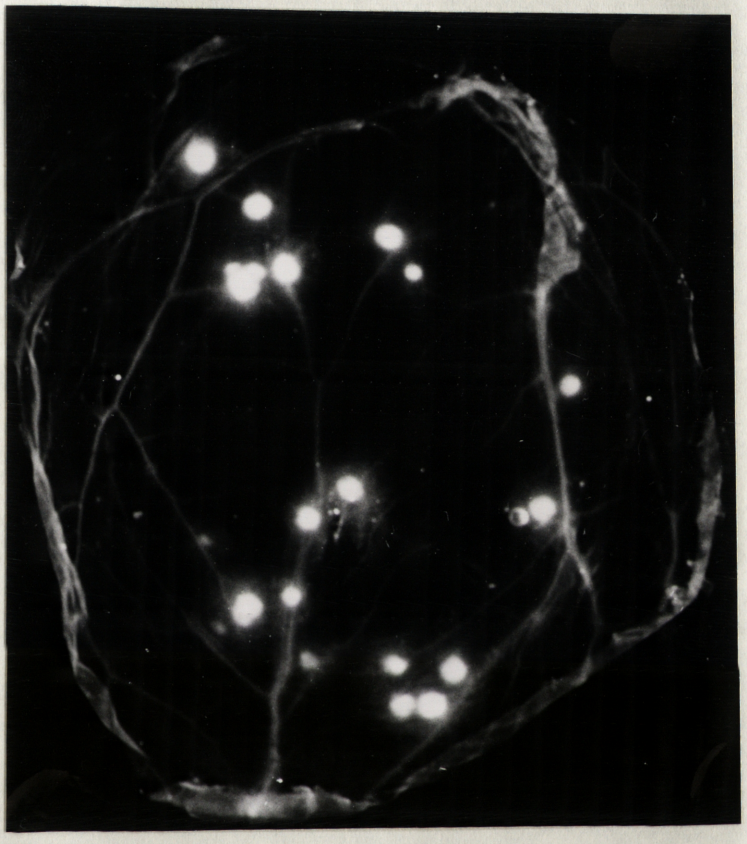


Figure 3.3. w8 of Group III.

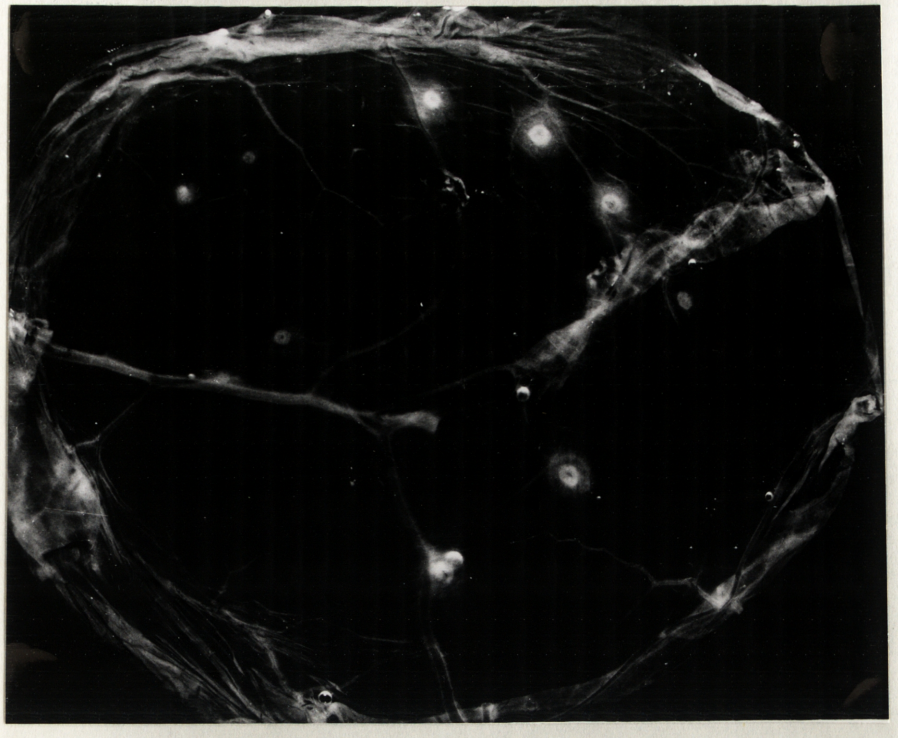


Figure 3.4. w11 of Group IV.

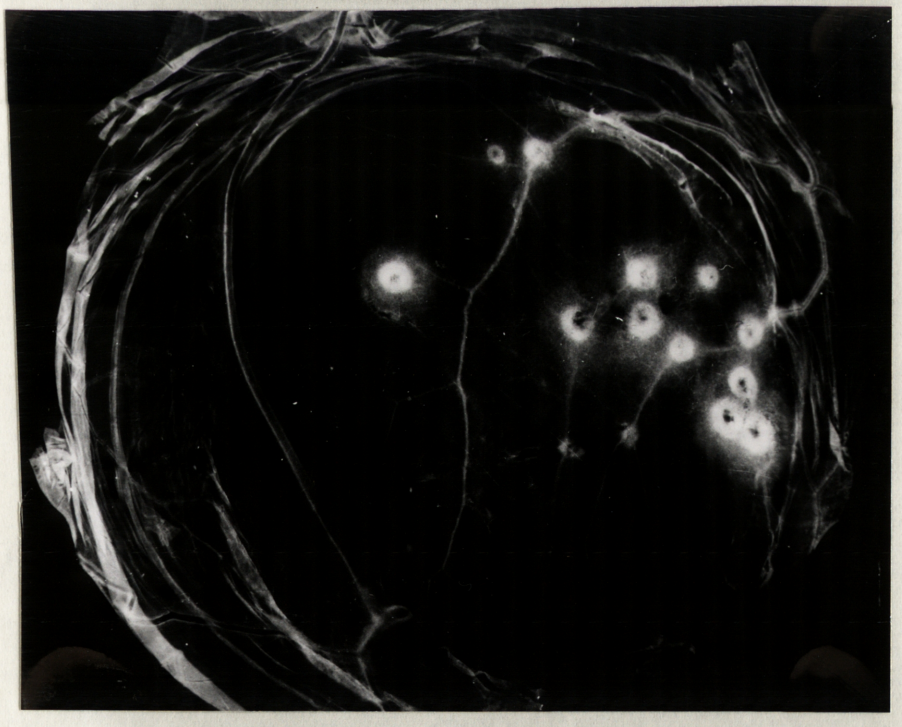
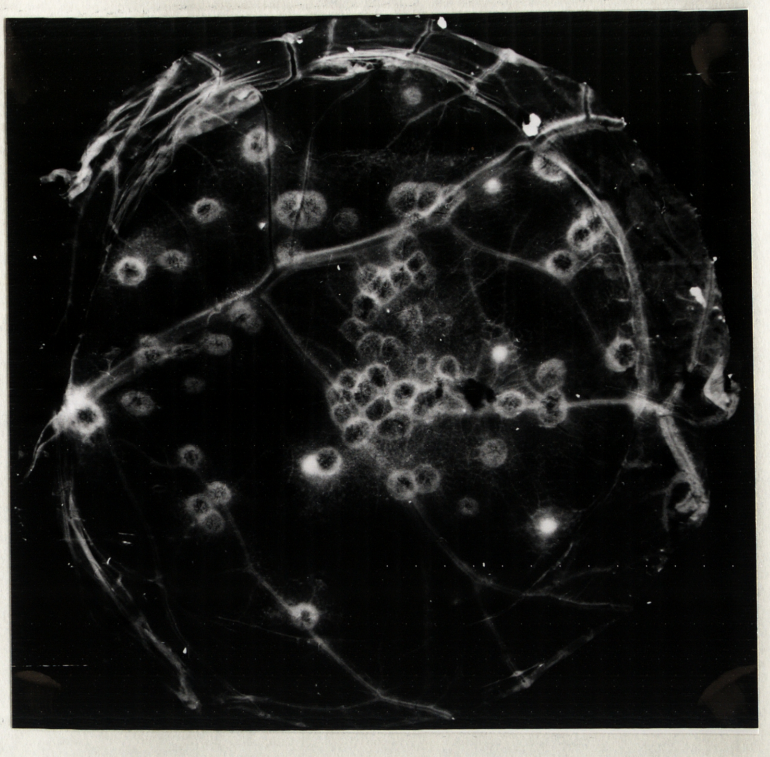
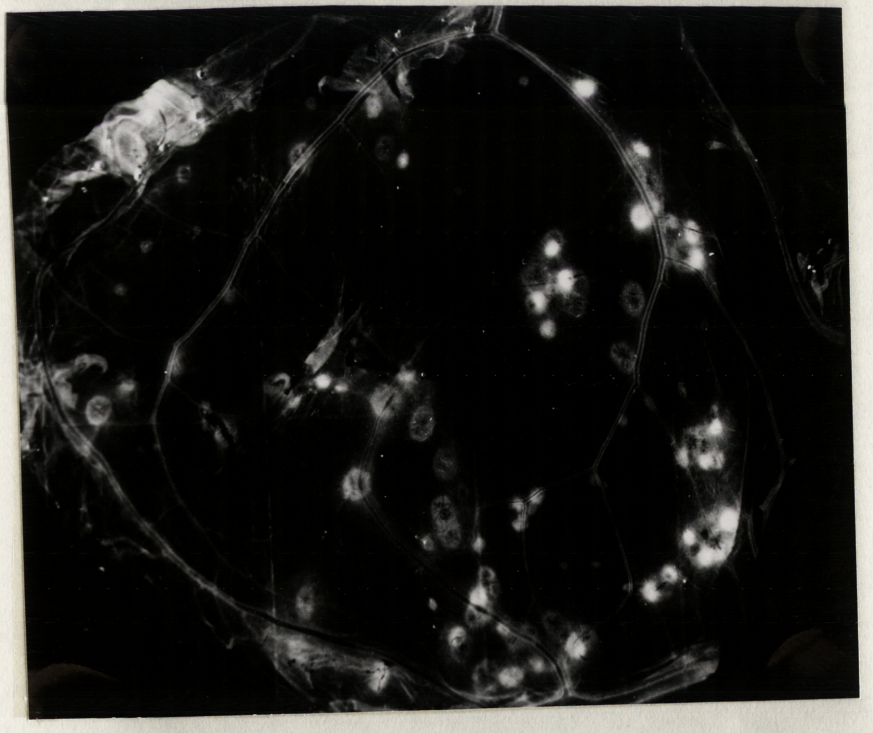


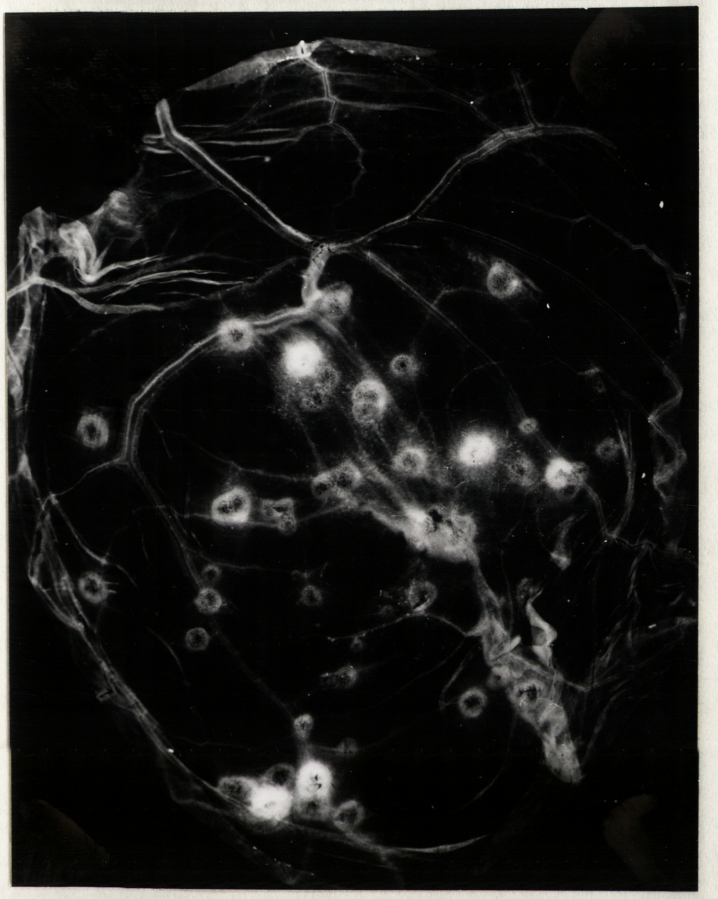
Figure 3.5. w18 of Group V.



RPU + w1.



RPU + w7.



RPU + w18.

Isolation of the white pocks was made by means of needles (as described in the appendix), and purification effected by two single pock passages. In this way nineteen different pocks of RPU-w were obtained.

Although on the primary membrane all the white pocks appeared identical (perhaps due to the crowding effect of the much larger number of red pocks) on purification they could be divided into five groups depending on the morphology of the pock. Representative morphological types of white pocks are illustrated in Figures 3.1 -3.5.

4. Examination of a number of properties of the white variants.

A number of other properties of these white variants were examined, namely their heat resistance, the production of haemagglutinin, their virulence for mice, the type of lesion produced in the rabbit skin, and the type of plaque produced on monolayers of chick embryo fibroblasts. The efficiency of plating (eop) on chick embryo fibroblasts was compared with the eop on the CAM. Methods used in the examination of these characters are described in the appendix.

The results are tabulated in Table VIII, in which are included for comparison, the properties of the parental RPU and the original white variant RPU-w described by Fenner. The table is divided into five groups based on pock morphology. Group I contains two variants which produced very small white pocks on the CAM, Group II consists of five variants, which produced small white pocks simi-

TABLE VIII.

Some biological characters of 19 separately isolated white variants of RPU, of RPU itself, and of RPU-w, the original white variant of RPU

Group	Number	Appearance of pock	Haemagglutinin titre	% Deaths in mice after i/c inoculation of 10^5 pfu	Type of lesion on rabbit's back after i/d inoculation of 10^5 pfu	No. Plaque forming units / No. Pock forming units	Size of Plaque
RPU		large, red	0	100%	large, haemorrhagic	1/3	large
RPU-w (original)		small, white	0	0%	small, nodule	1/8	small
1.	w1	very small, white	0	0%	small, nodule	-	very small
	w2	very small, white	0	10%	small, nodule	0	not visible
2.	w3	small, white	0	10%	small, nodule	1/5	large
	w4	small, white	0	0%	small, nodule	1/1	large
	w5	small, white	0	0%	small, nodule	-	large
	w6	small, white	0	0%	small, nodule	1/6	large
	w7	small, white	0	0%	small, nodule	1/2	small with irregular edg
3.	w8	large, white	2.5	20%	very small nodule	1/1.3	small
4.	w9	small white,	0	40%	small, nodule	1/5	small
	w10	with red	0	20%	small, nodule	1/3	small
	w11	centre	0	10%	small, nodule	-	small
	w12	" " "	0	10%	small, nodule	1/5	small
	w13	" " "	0	50%	small, nodule	1/6	small
	w14	" " "	0	90%	small, nodule	1/4	large
5.	w15	large white,	0	0%	small, nodule	-	large
	w16	with red centre	0	0%	intermediate size haemorrhagic	-	large
	w17	" " "	0	20%	small, nodule	-	large
	w18	" " "	0	100%	large haemorrhagic	1/2.5	large
	w19	" " "	0	100%	small, nodule	1/3	large

lar to those produced by the original white variant of Fenner's and Group III contains the only white variant isolated which produced large white pocks. The six white variants in Group IV produced small white pocks with red centres and the five variants belonging to Group V formed large white pocks with red centres. The results of the examination of their properties showed differences other than pock morphology among the Groups.

Group I. w1 and w2 could be distinguished from each other as w2 did not produce plaques on chick fibroblast monolayers even when large amounts of virus were plated. w1 could also be distinguished from the variants of the other Groups by the size of its plaques as well as by the morphology of its pocks. It produced much smaller plaques than the others.

Group II. The only variant to differ within this Group was w7 which produced small plaques with irregular edges whereas all the others produced larger plaques with regular edges.

Group III. Only one variant belonged to this Group and it was the only one to produce haemagglutinin. The original parental stock RPU does not produce haemagglutinin.

Group IV. The only variant to differ within this Group was w14 which was more virulent for mice than the others of this Group.

Group V. All the five variants in this group differed from each other. w15 and w16 were both a-virulent for mice but could be distinguished as w16 produced an intermediate type of lesion in rabbit skin after intradermal inoculation. w17 was of intermediate virulence for mice and w18 and w19 were of high virulence for mice. Variants w18 and 19 were different however, as w18 produced a haemorrhagic lesion on the rabbit's back similar to that of RPU and w19 produced a nodule like that of the other variants.

The heat resistance of all the variants was similar to that of RPU and the titre dropped approximately one log. after being heated at 55°C for 40 minutes.

The variants w5, w9, w10, w11, w14, w15, w16, w17 and w18 were "unstable", that is some of the pocks formed by their progeny were similar in appearance to the parental RPU. This property distinguishes w5 from w3, w4 and w6 of the same group. Several of the variants were indistinguishable from each other on the basis of the examination of the characters set out in Table VIII, these were w3, w4 and w6 of Group II; and w9, w10, w11;and w12 and w13 of Group IV.

The results of this analysis of characters of the different white variants of RPU show that clones originally classified together as "white variants" may differ in many properties. Not only do they produce different pocks on the CAM, but they also differ in the type

of plaque produced on chick embryo fibroblast monolayers, in their virulence for mice and rabbits and in their ability to produce haemagglutinin.

A simple interpretation to account for all these differences is the hypothesis that there are many different mutations of RPU which will give rise to clones of virus sharing the property of producing white pocks on the CAM. The correctness of this interpretation is confirmed in the next section, where it is shown that different white variants can recombine to produce the original parental strain RPU.

5. Genetic recombination between White Variants.

The properties of the white variants of RPU, namely their high frequency, and differences which are seen on detailed examination of their characters, make it a reasonable hypothesis that the "white" character of RPU is analogous to the r character of T2 and T4 bacteriophage mutants, that is, both are phenotypic expressions of a large number of different single step mutations of the parental virus.

If this is so, it should be possible to cross different white variants of RPU and produce parental RPU (wild type) as one of the products of recombination. Experiments were therefore carried out to see whether recombination would occur, and if so whether the results could be used with RPU as with T4, for genetic mapping.

Nine of the ninteen white variants produced varying proptions of pocks similar in appearance to wild type among their progeny. These red "revertant" pocks proved to be unstable too unlike the wild type.

Although these unstable variants are of considerable interest they were unsuitable for the recombination experiments as the appearance of wild type on the CAM was to be used as evidence of recombination. The ten stable variants were crossed in all possible pairs.

The recombination experiment was performed in HeLa cell monolayers grown in small screw-capped bottles. The technical details are described in the appendix. Care was taken to ensure adequate and as far as possible, equal multiplicity of infection, by adding a large dose of virus, approximately 100 pfu of each type of variant per cell (10^7 pfu of both variants in one ml of growth medium). Absorption was allowed to occur for two hours, then the cell layers were washed four times with Hank's balanced salt solution and one ml of growth medium was added.

After incubation for twenty four hours, the cells were placed at - 70°C and kept at that temperature until they could be titrated for pock type. To release the virus from the cells, the cells were thawed and sonicated using a 50 Watt Mullard sonicator for

thirty seconds. By this time all the cells were disrupted. The progeny of the crosses were then titrated on CAMs at a dilution which would give between twenty and forty pocks. The results which are tabulated in Table IX show that recombinant wild type occurred among most of the crosses.

6. Mapping of the White Mutants.

The results showed conclusively that white pock formation is the phenotypic expression of a number of different genotypes. Because of the large variation in percentage of wild type on individual CAMs containing the same material, the percentage recombination could not be used to detect the distances between the loci. These results could be used to arrange the white mutants linearly in positions determined by the occurrence or non-occurrence of recombinants between the different pairs. w13 produced wild type recombinants with all the other mutants except w1 and w2 but w1 and w2 differed from w13 in that they did not give wild type with w8 and w19. On the basis of these results w1 and w2, and w8 and w19 were indistinguishable. w3, w4, w6 and w7 produced large numbers of wild type recombinants when mated with the other six mutants, but not with each other. w7 was distinguishable from w3, w4 and w6 as it did not form recombinant wild type when mated with w12.

From these results three groups were formed within which the mutants could not be distinguished as a result of the recombination

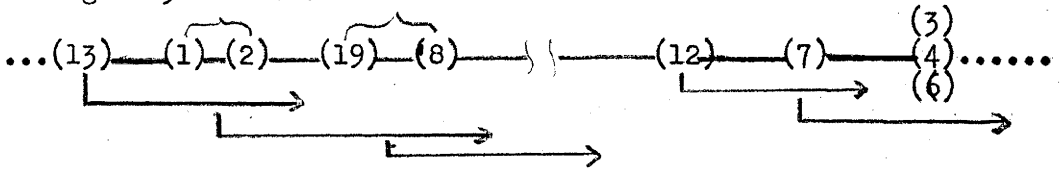
TABLE IX

Results of Genetic Crosses between the White Variants

$\left(\frac{\text{No. red pocks}}{\text{Total No. pocks}} \text{ counted}\right)$

	w1	w2	w3	w4	w6	w7	w8	w12	w13	w19
w1	0/150	0/400	144/354	89/283	44/136	66/149	0/493	3/31	0/300	0/239
	w2	0/150	30/189	42/170	15/129	23/95	0/432	13/103	0/271	0/173
		w3	0/100	0/604	0/297	0/505	10/311	35/78	6/135	50/100
			w4	0/200	0/438	0/254	45/314	18/124	10/69	14/75
				w6	0/103	0/580	18/124	42/155	21/113	20/144
					w7	0/85	19/125	0/250	6/153	43/158
						w8	0/113	7/271	3/116	0/198
							w12	0/200	8/163	3/408
								w13	0/112	3/397

experiment. These were w1 and w2, w8 and w19, and w3, w4 and w6. As differences in other characters had been detected between w1 and w2, and w8 and w19, these were placed at closely adjacent loci rather than at the same locus. This left w3, w4 and w6 as the only indistinguishable group of white mutants. A map of the white mutants is shown in Figure 3.6 below.



The position of the arrow-head indicates that recombinants have been detected with the loci to the right of it.

To detect any wild type recombinants which may have occurred with a smaller frequency, progeny of the crosses for which wild type had not been observed were titrated on a larger number of CAMs (these results are incorporated in Table IX)and were also inoculated undiluted into the brains of five week old mice. This latter method used the mouse brain as a selective environment for any virulent recombinants, such as wild type, which may have formed in the HeLa cell system. Duplicate brains were removed on the third day after inoculation as this was the time when the virus titre of the brain was highest. The brains were ground and titrated as described in the appendix. The results are summarised in Table X.

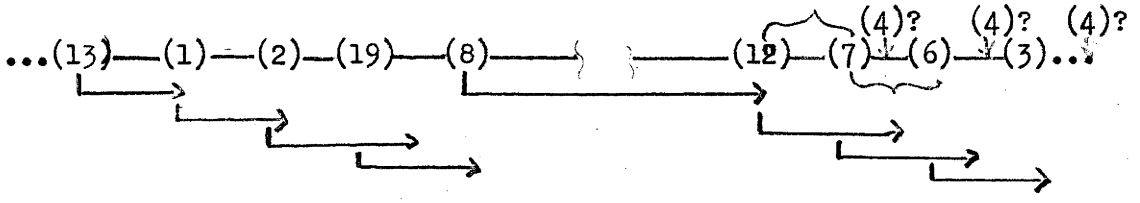
w4 produced red pocks after inoculation into the mouse brain, so the occurrence of wild type after brain inoculation in crosses containing it, could not be used as evidence of recombination. This property distinguished w4 from the other mutants.

TABLE X.

Appearance of wild type in the crosses after using the selective environment of the mouse brain.

Progeny of Crosses.		Occurrence of Wild Type.
	1 x 2	yes
	1 x 8	"
	1 x 13	"
	1 x 19	"
	2 x 8	"
	2 x 13	"
	2 x 19	no
	3 x 6	yes
	3 x 7	"
	6 x 7	no
	7 x 12	no
	8 x 19	yes
Controls	1	no
	2	"
	3	"
	4	yes
	6	no
	7	"
	9	"
	12	"
	13	"
	19	"

Differences had now been detected between all ten mutants which meant that all ten mutations to white pock form had occurred at separate loci in the genome of RPU. Although no recombinants had been observed among the progeny of crosses w2 x w19, w6 x w7 and w7 x w12, and the individual orders of w3, w4 and w6 could not be placed on the map, a more complete map could now be drawn to describe the positions of the mutations. Such a map is shown in Figure 3.7 below.



Several of the wild type formed by recombination were purified and tested for mouse virulence and the type of lesion they formed in rabbit skin. Their virulence and lesions were similar to those of the original wild type. All the progeny of the recombinant wild type also formed red pocks.

Discussion.

1. Recombination between white mutants.

Preliminary experiments showed that it was possible to distinguish a small number of white pocks on a CAM containing a majority of red pocks, reasonably accurately, and also that the white mutants which occurred with a high frequency were not host-induced.

A number of the white mutants were not stable, that is, they produced varying numbers of pocks similar in appearance to wild type, among their progeny. These red pocks were purified but were unstable too, unlike the original wild type or the wild type formed as a result of genetic recombination. The unstable mutants could not be used for recombination experiments.

The white mutants proved to be genetically different. This was shown by a). differences occurring among their known markers and b). as a result of genetic recombination when wild type particles were produced.

These results indicated that there are a number of distinct loci controlling red pock formation in RPU. This situation may be compared with that occurring for the plaque-type mutants of the T2 and T4 bacteriophages. Hershey and Rotman (1948, 1949), Doermann and Hill (1953) and Benzer (1955, 1957) showed that there were a large number of distinct loci controlling plaque formation in these bacteriophages.

2. Construction of a Map of the White Mutants.

A technique for detecting accurately the number of recombinant wild type formed in any cross was not available because of lack of sufficiently accurate assays. As considerable variation occurred in the percentage of red pocks appearing on individual CAMs containing the same material, the percentage recombination used for indicating distances between loci could not be used and a map similar to that obtained for the r mutants of T2 and T4 could not be plotted.

The ten mutants could be plotted on a one dimensional diagram where each mutant was represented by a point, and relative distances were determined depending on how easily the wild type recombinants were detected, that is, whether they were detected on a small number of CAMs, a larger number of CAMs, or only after selection in the mouse brain.

The fact that all ten mutants were different suggest that there are many loci controlling RPU formation. If there are N different ways (that is N loci) of getting red to white, and one assumes that each of these ways are equally likely, them the probability that each of the x whites represents a mutation at a different locus

$$Px = \frac{N(N-1)(N-2).....(N-x+1)}{N^x}$$

$$\text{Log } Px = -\frac{x(x-1)}{2N}$$ (Feller 1950).

In this case x = 10

$$\text{therefore } \log Px = \frac{-90}{2N} = \frac{-45}{N}$$

$$\text{therefore } N = \frac{-45}{\log Px} = \frac{45}{\log(1/Px)}$$

$$\text{For } P = 0.5 \quad N = \frac{45}{\log 2} = \frac{45}{0.3} = 150$$

$$\text{For } P = 0.05 \quad N = \frac{45}{\log 20} = \frac{45}{1.3} = 35$$

Thus if there were only thirty or forty loci, it would be very unlikely that the first ten white mutants recovered from a single clone of RPU would be different. It is probable therefore, that there are many more loci than this.

The white mutants arise with a frequency of about $10^{-1.7}$. If the mutation at each locus causing mutation to white, occurs at a frequency of 10^{-5} (probably an upper limit to the likely mean frequency) then the number of loci must be $10^{-1.7} / 10^{-5} = 10^{3.3}$ to provide an overall rate of $10^{-1.7}$.

Summary.

There are at least ten loci in the genome of RPU which control red pock formation. This has been shown by crossing ten white mutants and obtaining wild type as a result of genetic recombination. These results enabled all ten to be separated and a genetic map to be constructed.

Calculations based on the results suggest that there could be between fifty and 2,000 loci controlling red pock formation.

General Summary.

It was hoped that adaptation of dermal strains of vaccinia virus to the mouse brain would be associated with a change in pock morphology so that the chorioallantoic membrane could be used to elucidate the process of adaptation. Differences in pock morphology had already been shown between neurovaccinia and dermal vaccinia strains and also between CL (a strain of very low virulence) and its mouse-virulent mutant, CL-r. Two strains out of the ten used, became adapted to the mouse brain after five serial mouse brain passages but their increase in virulence was not accompanied by a change in pock morphology. Because the chorioallantoic membrane could not be used to follow their process of adaptation the investigations were not completed.

The final and important part of this work was concerned with the white variants of a strain of rabbitpox. White variants are produced by all red pock producing pox viruses which have been investigated. They have been shown to occur with very high frequencies which suggested that a white pock may be the common phenotypic expression of a number of different genotypes. Recombination experiments have shown that this is so and as a result a genetic map was constructed for ten white mutants of RPU. As there was no accurate assay system to determine the number of wild type recombinants formed the distance between the loci could not be determined;

relative distances could be determined which enabled all ten mutants to be placed in different positions on the map.

Although parts of the genetic structure of some bacterial viruses have been mapped in considerable detail, it has not been possible to construct a genetic map for an animal virus. These experiments make a start in the construction of a map of the genome of vaccinia virus.

APPENDIX.

This appendix contains details of the materials and methods used in the experiments.

I. Composition of the Solutions Used.

1. Gelatin Saline;

0.8% NaCl

0.006% $CaCl_2.6H_2O$

0.017% $MgCl_2.6H_2O$

0.12% H_3BO_3

0.005% $Na_2B_4O_7.10H_2O$

0.5% "Fine-leaf gelatin E"

This medium, after sterilization by autoclaving, was used for making suspensions of brain material and confluent membrane material, and as a diluent for infectivity tests.

2. Calcium-Magnesium Saline.

0.8% NaCl

0.006% $CaCl_2.6H_2O$

0.017% $MgCl_2.H_2O$

0.12% H_3BO_3

0.005% $Na_2B_4O_7.10H_2O$

This solution was used for haemagglutination tests.

3. M^{c}Ilvaine's Buffer (modified). (M^{c}Ilvaine 1921)

18 ml. M/5 Na_2HPO_4

1.83ml. M/10 Citric Acid

1.5 gm. Gelatin

7.4 pH and distilled water to 1 litre.

4. Puck's Saline A. (Marcus, Cieciura, Puck 1956)

Phenol red	0.02 gm
NaCl	8.0 gm
KCl	0.4 gm
Glucose	1.0 gm
$NaHCO_3$	0.35 gm
Twice distilled water	1.0 litre

Sterilized by autoclaving and stored in the refrigerator.

5. Puck's Trypsin solution.

Trypsin (1:250 Nutri Difco)	5.0 gm
Saline A	100.0 ml

Dissolved with stirring for an hour, and sterilized by Seitz D-9 filtration. Stored frozen.

6. Earle's Saline (10x concentrated). (Earle 1943)

NaCl	72.0 gm
KCl	4.0 gm
$CaCl_2$	2.0 gm
$MgSO_4.7H_2O$	2.0 gm
$NaH_2PO_4.2H_2O$	1.41 gm
Glucose	45.0 gm
Water	1,000 ml.

Modified from original Earle's, (glucose). Sterilized by autoclaving and stored at 4°C. To make 1,000 ml of single-fold Earle's saline 740 ml distilled water is added to 100 ml 10x concentrated Earle's

saline and antibiotics and 160 ml of 1.4% sodium bicarbonate are added.

7. Eagle's Basic Medium. (10x concentrated) (Eagle 1955).

Quantities in mg/l of 10x concentrated solution.

Amino Acids

Arginine	200
Cysteine.HCl	35
Histidine.HCl	40
Isoleucine	26
Leucine	180
Methionine	75
Phenylalinine	40
Threonine	120
Tryptophane	20
Tyrosine	180
Valine	120
Glutamine	1475

Vitamins

p-aminobenzoic acid	1
Biotin	10
Choline	10
Folic Acid	10
Nicotinic Acid	10
Nicotinamide	10
Pantothenic Acid	10
Pyridoxal	10
Pyridoxamine	10
Riboflavin	1
Thiamin	10

Salts (g/l of 10x concentrated)

Sodium chloride	60
Potassium chloride	3
Magnesium sulphate	1.5
Calcium chloride	1.05 (anh.)
Sod. Phosphate ($Na_2HPO_4.2H_2O$)	0.45
Pot. Phosphate (KH_2PO_4)	0.45
Ferric Nitrate	1 mg
Glucose	15 g/l of 10x conc.
Phenol red	o.2 g/l of 10x conc.

8. Chick Fibroblast Growth Medium.

Eagle's basic medium (single concentration) sterilized by Seitz filtration, after the addition of 0.25 ml concentrated HCl per litre.

50 ml. of 1.4% sterile sodium bicarbinate per litre

10 - 20% calf serum sterilized by Seiz filtration and heated at 56°C for 30 minutes to destroy vaccinial inhibitors.

10 ml of a solution of penicillin and streptomycin/l containing 100 units of penicillin and 100 µg of streptomycin per ml.

9. Nutrient Agar Overlay (for chick fibroblast monolayers). (Franklin et al.,1957).

0.5% Lactalbumin hydrolysate (enzymatic)
0.1% yeast extract (Difco)
0.1% crystallized Bovine plasma albumin
8.3% by volume of chick embryo extract
0.85% unwashed Difco agar
in Earle's Saline (single concentration).

10. Preparation of Chick Embryo Extract. (Bazeley and Thayer 1954)

9-11 day old whole chick embryos were harvested aseptically into a sterile Atomix containing an equal volume per embryo of Hank's balanced salt solution. This was mixed at full speed for three minutes, then left to stand at room temperature for thirty minutes. the suspension was then centrifuged at 2,500 rpm for thirty minutes and the supernatant carefully pipetted off and distributed in 10 ml quantities and rapidly frozen in an alcohol dry-ice bath. The extract was stored at -20°C and before use thawed and clarified by centrifugation at 3,000 rpm for thirty minutes.

11. Hank's Balanced Salt Solution (BSS). (Bazeley and Thayer 1954)

NaCl	8.0 g/l	$CaCl_2$	0.14 g/l
KCl	0.4	Na_2HPO_4	0.06
$MgSO_4.7H_2O$	0.1	KH_2PO_4	0.06
$MgCl_2.6H_2O$	0.1	Glucose	1.0

5 ml of 0.4% aqueous phenol red solution was added for each litre and BSS was sterilized by autoclaving at 10 lb pressure for ten minutes. Prior to use antibiotics and three ml of sterile 1.4% sodium bicarbonate were added to 100 ml of BSS.

12. Dulbecco's Phosphate Buffered Saline. (Dulbecco and Vogt 1954).

(A)	NaCl	8.0 gm	(B)	$CaCl_2$	0.1 gm
	KCl	0.2 gm		Water	100 ml
	Na_2HPO_4	1.15 gm			
	KH_2PO_4	0.2 gm	(C)	$MgCl_2.6H_2O$	0.1 gm
	Water	800 ml		Water	100 ml

(A), (B) and (C) were autoclaved separately.

13. HeLa Cell Growth Medium.

0.5% Lactalbumin hydrolysate.
20% Calf serum (heated at 56°C for 30 minutes to destroy vaccinial inhibitors)
in Hank's BSS.

II. Virus Strains.

The origins and properties of the strains used and most of the technical methods have already been described in an earlier paper by Fenner (1958) and are as follows:

1. Vaccinia-Connaught Laboratory (CL). Connaught Laboratory strain, probably derived originally from New York City Board of Health strain (Parker et al.,1941). Used by Craigie (1932) and then by the Rockefeller Institute Group (Smadel and Hoagland 1942). Maintained by serial passage in rabbit skin (scarified). Passed nine times in rabbit testis by Dr. Duran-Reynals and then fifteen times on chorioallantoic membrane by Dr. B.A. Briody.

2. Vaccinia CL-r. A red pock forming mutant of CL recovered from a mouse brain by my colleague Miss G..Woodroofe.

3. Rabbitpox Utrecht (RPU). Originated from a spontaneous outbreak of rabbitpox in Utrecht, Holland, in 1941 (Jansen 1941, 1946). Original strain received by Dr. Dekking (Amsterdam , in 1950. Passed once on eggs and freeze-dried until 1951, then passed twelve times intracerebrally in mice before being sent to Dr. Gispen.

4. Rabbitpox Utrecht-white (RPU-w). White variant derived from 3.

5. Vaccinia Noguchi (Nog). A derivative of Noguchi's testicular virus (Noguchi 1915) sent from the Rockefeller Institute to Dr. Duran-Reynals, who passed it seven times in rabbit testis, and thence to Dr. B.A. Briody, who passed it fifteen times on the chorioallantoic membrane.

6. Vaccinia international Health Division (IHD). Propagated for many passages by intracerebral inoculation of mice (Parker et al.,1941, Thompson et al.,1953), then passed once on the chorioallantoic membrane.

7. Vaccinia International Health Division-white (IHD-w). White variant derived from 6.

8. Vaccinia Hall Institute (HI). A derivative of the Pasteur Institute strain (PI), supplied to the Walter and Eliza Hall Institute by Dr. A. Galloway in 1934 (Keogh 1936). After an initial passage in rabbit brain it has been maintained entirely by passage on the chorioallantoic membrane.

9. Vaccinia Hall Institute-w (HI-w). White variant derived from 8.

10. Vaccinia Williamsport (WP). Recovered from a child (Minton et al., 1953). Human material passed once in rabbit skin, once in rabbit testis and once on the chorioallantoic membrane.

11. Vaccinia Nelson (Nel). Derived from a strain found by Nelson (1938) to induce fatal infection in mice after intranasal inoculation. Passed four times on the chorioallantoic membrane by Dr. G. Rake and fifteen times by Dr. B.A. Briody.

12. Vaccinia Mill Hill (MH). Supplied as elementary body preparation (Mill Hill EB 102) from scarified rabbit back 1952. Since then passed several times by scarification of rabbits.

13. Vaccinia Lederle 7N (7N). Originally derived from scabs of human vaccinations obtained in 1909 from the New York City Board of

Health and since maintained in the Lederle Laboratories by irregular alternate passages through calves, rabbits and men and then seven passages intradermally in rabbits followed by three passages on the chorioallantoic membrane.

14. Vaccinia Lederle-red (Led-r). The component of a strain which had been derived from human vaccination scabs (7N) and since been maintained by irregular alternate passages through calves, rabbits and men.

15. Vaccinia Lederle-white (Led-w). The white variant from the material derived from the human vaccination scabs.

16. Vaccinia Pasteur Institute (PI). The original neurovaccinia strain of Levaditi and Nicolau (1923). Since then isolation has been maintained by rabbit brain passage, except for variable intervals of storage in glycerol in the refrigerator, or in lyophilized state.

17. Vaccinia Gillard (Gill). Recovered by Burnet from a commercial lot of vaccine lymph brought to Australia by the United States Army Medical Service in 1942. Selected because of good pock production on the chorioallantoic membrane and maintained at the Walter and Eliza Hall Institute of Medical Research by serial passage on the chorio-allantoic membrane.

18. Cowpox Amsterdam-white (CPA-w). A white variant of a cowpox strain sent to Dr. Gispen (Utrecht) by Dr. F. Dekking (Amsterdam) which was maintained in duck embryos. (Gispen 1955).

19. Cowpox Brighton-white (CPLB-w). A white variant of a cowpox strain isolated from lesions of a cowman's hands on a farm in Brighton in 1937 (Downie 1939) which was passed by pad inoculation in two guinea pigs, and then fifteen to twenty times in rabbit skin, and twice in chick embryos.

III. Experimental Animals.

1. Eggs.

The eggs used for virus titrations, for single pock isolation and for making virus stocks, were white Leghorn or a cross between white Leghorn and Australorp.

2. Mice.

The mice were bred in the University Animal Breeding Establishment from stock originating from the Walter and Eliza Hall Institute, Melbourne.

3. Rabbits.

The rabbits were bred in the Animal Breeding Establishment of the University and were ones which had originally been used for myxomatosis experiments and had since recovered. Their age was approximately six months.

IV. Technical Methods.

1. Infectivity titrations.

These were made by pock counts on the chorioallantoic membrane of 11 or 12 day old chick embryos. The CAMs were inoculated with 0.05 ml of the appropriate dilution according to the method of Beveridge and Burnet (1946). Pocks were counted after two days incubation (3 days for cowpox) of the inoculated eggs at 36°C. Infective titres were expressed as pock forming units (pfu) per ml.

2. Single Pock Isolations.

For isolation and purification of single pocks a needle technique was used. Virus was removed from the pock by pricking its centre with a fine sterile needle which was then washed in one ml of gelatin saline. This suspension was then sonicated using a 50 Watt Mullard sonicator to free the virus from the cells. The SPs were put through two passages as a means of purifying the virus.

3. Preparation of Suspensions of Virus from Infected Materials.

a. Grinding.

CAMs and mouse brains reaped under sterile conditions, were cooled in the refrigerator and then placed in small porcelain mortars which had been chilled to -20°C. The tissues usually froze immediately and were ground without added abrasive with a chilled pestle until they formed a paste. This material was suspended in gelatin saline

(one ml for each brain or membrane) and centrifuged at 3,000 rpm to clarify. The supernatant fluid was used for all tests. Preliminary experiments have shown that there is no significant loss of virus during grinding. All viral suspensions were stored in a mechanical refrigerator operating at -65° C.

b). Genetron Extraction.

To obtain high titres of semipurified virus suspensions, a modification of the method of Gessler et al., (1956) for isolating viruses by selective fluorocarbon deproteinization, was used.

Five ml of Genetron (trichlorotrifluoroethane) and ten ml of dilute McIlvaine's citrate buffer were added for every four confluent membranes, and these were homogeneised in a Virtis homogeneiser surrounded by ice, for five minutes at 27,000 rpm. The milk-like homogenate was centrifuged at 2,000 rpm in a refrigerated centrifuge during which time it separated into three sharp layers. The aqueous middle layer was carefully removed and saved. In order to further liberate large amounts of virus, 10 ml of dilute buffer solution were added to the top and bottom layers and this was homogeneised again for five minutes at 27,000 rpm. The second homogenate was centrifuged at 2,000 rpm for five minutes and then the middle clear aqueous portion was removed carefully and added to the first aqueous portion. Five ml of genetron was added to this and a third homogeneisation was performed and centrifuged at 2,000 rpm for five minutes. The water-clear super-

natant which resulted, contained the viruses in a pure state free from lipid and non-viral protein contained in the same tissue.

To concentrate the virus it was spun down in a Spinco centrifuge at 14,000 rpm for ten minutes and resuspended in a very small volume of gelatin saline.

4. Intracerebral Inoculation of Mice.

Groups of 5 - 6 weeks old mice were inoculated intracerebrally under ether-chloroform anaesthesia, with virus suspensions containing 10^5 pfu in a volume of 0.03 ml. In the growth of vaccinia virus in the mouse brain duplicate brains were removed under sterile conditions, at the appropriate times, and ground and titrated as previously described.

5. Intradermal Inoculation of Rabbits.

Rabbit skin lesions produced by the vaccinia virus strains were compared by inoculating doses of 0.1 ml containing 10^5 pfu of virus intradermally in the shaved backs of rabbits and recording the character and size of the resultant lesions five days later. Ten to fifteen inoculations including controls were made on each back.

6. Haemagglutinin Titrations.

Haemagglutinin titrations were carried out in plastic trays, using Takatsy's loop (Takátsy 1955). Dilutions of infected material were carried out in calcium-magnesium saline containing 1% normal rabbit serum to inhibit lipid agglutination (Stone 1946). A drop of 5% suspension of vaccinia-susceptible fowl red blood cells was added

to give a final concentration of 0.5% cells, and after shaking, the trays were incubated at 37°C for twenty minutes, shaken again, replaced in the incubator, and read forty minutes later, partial agglutination being the end-point (Nagler 1942).

7. Heat Resistance Tests.

These tests were made by completely immersing approximately 0.1 ml of confluent CAM suspensions in sealed glass capillaries in a water bath at 55°C. Pock counts were made with replicate ampoules before heating and after heating for forty minutes.

8. Limit Dilution Technique.

This technique was applied to vaccinia virus inoculated into brains of mice and intradermally into rabbits' backs. It consisted of inoculating dilutions of the virus material in two-fold steps into intracerebrally into a number of mice or intradermally into a number of rabbits' backs. The limit dilution was the highest dilution which caused the formation of a nodule on the rabbits' backs or the death of one mouse out of ten inoculated.

This nodule was sliced off with a sharp sterile scalpel and ground with fine sterile sand in a mortar with a pestle until it formed a paste. This material was then suspended in one ml of gelatin saline and sonicated. The brain was ground and titrated as previously described.

9. Tissue Culture.

a. The Preparation of Chick Fibroblast Monolayers.

The fibroblasts were prepared using a modification of Dulbecco's method (1952). 10 - 12-day old chick embryos minus heads and legs, were harvested aseptically into Puck's saline. The embryos were then cut into 4 - 6 pieces and washed once in cold Puck's and once in Puck's saline warmed to 37°C, to remove red blood cells. The saline was then removed and 3 - 5 ml of 0.25% Trypsin per embryo was added, and this suspension was stirred on a magnetic stirrer in a 36°C room for fifteen minutes. The trypsinized cells were removed and placed in an ice-bath and the trypsinization process was repeated with the remaining embryo pieces for a further fifteen minutes. The two trypsinized suspensions were then pooled and filtered through 3 - 4 layers of coarse gauze and the resultant filtrate was spun at 1,800 rpm for four minutes. The trypsin was poured off, the cells resuspended in Puck's saline and spun again and finally suspended in 10 - 15 ml of Puck's saline.

A cell count was made of the final suspension using a haemocytometer ($1 - 5 \times 10^8$ cells were usually obtained from each embryo) and 3×10^7 cells were added to each 70mm plate together with ten ml of the growth medium. The plates were incubated for 1 - 2 days in a 7.5% CO_2 gassed and humidified incubator at a temperature of 36°C.

By this time a complete monolayer had formed and the medium

was removed and the plates washed twice with five ml of pre-warmed phosphate buffered saline. 0.5 ml of a virus suspension at the appropriate dilution in growth medium, was added, and absorption allowed to occur for three hours (experiments have shown that 50% absorption occurs in one hour and 100% in three hours) after which time six ml of nutrient agar overlay was poured on per plate. Overlays without chick embryo extract were tried but they proved unsatisfactory. Embryo extract appeared to be a necessary factor if the fibroblast monolayers were to remain viable long enough for the vaccinia virus to form plaques. Under the overlay used the cells remained viable for one to three weeks. The necessity of chick embryo extract for survival of fibroblasts for long time intervals, has been reported by Chaparas (1958) who used the monolayers for toxoplasma plaque production, and Waterson (1958) found that embryo extract when added to enriched-agar overlay increased the number of plaques formed by fowl plague virus.

After allowing the agar overlay to set, the plates were returned to the gassed and humidified incubator for three to five days. Plaques were visible macroscopically on the third day after infection and the maximum count of plaques occurred on the fifth day after infection. These results are similar to those obtained by Noyes (1953). The counts were made after first staining with five ml per plate of a 1/10,000 dilution of neutral red in phosphate buffered saline and incubating for two hours.

b. The Cultivation of HeLa Cells.

A cloned line of HeLa cells (Clone 4) kindly provided by my colleague Miss G. Woodroofe, were used for the recombination experiments. The cells were grown in medical flats in 10 ml of HeLa growth medium for 5 - 7 days, by which time a confluent layer of cells had formed on the side of the bottle. The cells were trypsinized off the bottle using 5 ml of 0.05% trypsin in Puck's saline and incubating for 5 minutes at 37°C. To prevent clumping, 10 ml of HeLa medium was added immediately before the cells were counted. Approximately 7 x 10^4 cells in one ml of CO_2-saturated HeLa medium were added to each of the required number of small screw-capped bottles which were then incubated upright until confluent cell layers had formed on the bottoms of the bottles (2 - 3 days). The medium was then removed and the cell layers washed once in one ml of Hank's balanced salt solution. The cells were then ready for infection.

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